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1. MacCorquodale, D. W.; Thayer, S. A., and Doisy, E. A.: J. Biol. Chem. 115: 435, 1936.

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ENDOCRINOLOGY

VOLUME 38 FEBRUARY, 1946 NUMBER 2

THE PERIPHERAL INHIBITORY INFLUENCE OF LARGE DOSES OF TESTOSTERONE ON EPIPHYSEAL CARTILAGINOUS GROWTH

MAX REISS, J. E. FERNANDES AND Y. M. L. GOLLA

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THE PRESENT INVESTIGATION arose from clinical experiences with hormonal therapy of acromegaly carried out in the last five years at this Institute (Hutton & Reiss 1942).

Several results of this treatment could not be explained, notably the very quick reaction several patients showed after the onset of treatment with oestrone and testosterone. Particularly noticeable in this connection was a patient who developed acute acromegaly after male climacteric changes had begun. His predominant pathological symptoms were—apart from the usual acromegalic symptoms—a very marked growth of the costal cartilages (the patient could not close his coat) and the growth of the thyroid cartilage, causing almost complete aphonia. After having daily injections of 50 mg. testosterone propionate for 10 days the patient's speech was almost completely normal, and the cartilaginous overgrowth of the ribs not only stopped, but regressed sufficiently to enable the patient to again button his coat. Such rapid regression could not possibly be explained by the inhibition of the increased production of growth hormone in the pituitary only.

In this case, in which a rapid amelioration was obtained by the administration of the male sex hormone, two explanations are possible; the testosterone might act peripherally by primarily influencing the cartilaginous overgrowth, or only by influencing the anterior lobe of the pituitary, or by both processes simultaneously.

In an attempt to solve this problem, hypophysectomized animals were used, with a view to determine whether the growth response to injection of growth hormone could be influenced by steroid hormones.

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METHODS

Measurement of growth: It was necessary to use a method where the actual growth of cartilage was measured as accurately as possible. Previous standardization methods—in which weight changes only were measured—were useless for this investigation, since an increase in total body weight may be the result of a multitude of factors such as, fat apposition, increase in water content of the tissues, etc., besides the actual growth.

Two methods are considered applicable in this direction. The first method was published by Freud, Levi & Kroon (1939) and was based on the radiographic measurement of the total length of tail of hypophysectomized rats. The other method was published recently by Evans, Simpson, Marx & Kibrick (1943), when only a microscopic measurement of the width of the proximal epiphyseal cartilage of the tibia was used.

For this particular investigation Freud's method was employed, since it offered many advantages compared with the other method. Thus the experimental animals need not be sacrificed, their growth can be radiologically measured in different stages of the experiment, and the measurement itself is considerably more exact than the measuring of the tibia epiphyseal cartilage, the thickness of which differs in various parts.

The observations by Freud's method were made with a generator (secondary current) of 40 to 60 k.v. (Sunic METALIX PORTABLE) which was used together with a film of fine grain high contrast, allowing a high accuracy of measurements (Ilford's Industrial X-ray film C. $8\frac{1}{2}'' \times 6''$ in size). Exposures were taken from a distance of 18″ of the X-ray tube for two seconds. Measurements were taken in mm. scale on the prints made from the films. At first an attempt was made to measure only the lengths of a certain number of vertebrae, but later it was found better to measure the total length of the tail from the first vertebrae, determined by a line tangential to the pelvic spinal. An attempt was made to use X-ray films of the tibia for measuring the width of the cartilage after magnification, and the length of the bone, but the occurrence of occasional distortion did not allow the desired accuracy.

Treatment of animals: Wistar rats bred at the Institute were used. They were fed on a mixed diet, very rich in vitamins, with dried milk, whole meal flour and maize meal as basic ingredients. The rats were kept throughout the experiment in a thermoregulated room at 28° C.

The hypophysectomy was carried out on rats weighing 110 gm. to 130 gm. by the usual parapharyngeal approach.

Growth hormone extracts were injected intraperitoneally; oily solutions of the steroid hormones were injected subcutaneously. Injections were started a day after hypophysectomy, since it was found in accordance with Freud that one week after hypophysectomy the rat tails were already very insensitive to growth hormone.

The animals were X-rayed under anaesthesia, thus making it possible to get 8 to 10 tails on a film.

Preparation of growth hormone extract: The growth hormone was produced from fresh frozen pituitary anterior lobes. They were finely minced and extracted in the ball mill with four times the volume of water at pH 8. The total extract was adjusted to pH 6, and centrifuged. The clear supernatant was adjusted to pH 4.1; the growth hormone settled nicely overnight in the icebox. The precipitate was redissolved and acetone up to a concentration of 35% was added. The inert precipitate falling out was removed and the fil-

trate precipitated with twice the volume of acetone. The precipitate was washed with acetone and dried. This extract proved quite active but still contained traces of gonadotrophic, thyrotrophic and corticotrophic hormones. For further purification it was redissolved in 20 volumes water, the solution cleared by centrifuging, the pH first adjusted to 5, the precipitate removed and then the growth hormone again precipitated at pH 4.1. This precipitate was again washed with acetone and dried. It did not contain any amount of other pituitary anterior lobe hormone, of gonadotrophic and thyrotrophic only very little, corticotrophic action was still present. 1 mgm. of the preparation was equivalent to about 1 gm. fresh pituitary.

Histological methods: For histological investigations the tibia were decalcified in a 1% phloroglucinol, 5% nitric acid solution and stained with Mallory's Anilin blue-orange G. stain.

RESULTS

The daily injection of 5 mgm. of the growth hormone extract (see Table 1) produced an average growth of 11 mm. in the tails of hypophysectomized rats, while the body weight was increased by 23%.

TABLE 1

No. of animals	Treatment with		Duration of experiment in days	Average growth of of tail $\pm\sigma$	Average change in body weight $\pm\sigma$
	Growth hormone	Steroid hormone			
12	Untreated controls.		14	0	7 ± 1.2
11	5 mgm. daily	—	14	11.5 ± 1.8	23 ± 3.4
7	—	4 mgm. Testosterone propionate daily.	14	0	11 ± 2.1
8	5 mgm. daily	4 mgm. Testosterone propionate daily.	14	4.1 ± 1.2	12 ± 2.25
6	5 mgm. daily	0.1 mgm. Oestrone daily.	14	2.2 ± 0.9	16 ± 2.1
5	5 mgm. daily	2 mgm. Desoxycorticosterone acetate daily.	14	7.1 ± 1.4	20 ± 1.8
6	3 mgm. daily	—	14	9.0 ± 1.1	16 ± 1.0
5	3 mgm. daily	22–27 mgm. implant Testosterone.	14	2.0 ± 1.05	10 ± 1.3

Testosterone propionate given in a dose of 4 mgm. daily, did not influence the tail lengths, neither did it influence the weight decrease usually seen after hypophysectomy. If given in combination with growth hormone this dose of testosterone showed a definite inhibiting effect on the growth action. The daily growth achieved with 5 mgm. of the growth hormone extract was reduced by more than 50% to only 4 mm. The body weight increase was also considerably smaller.

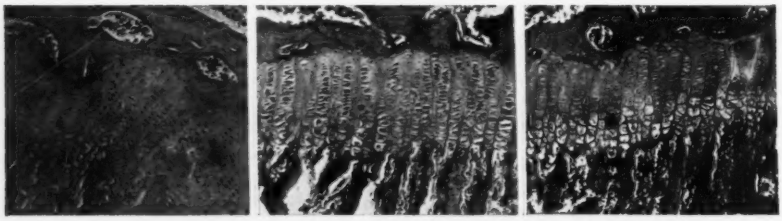
A single implantation of 22 mgm. to 27 mgm. testosterone also produced a very considerable inhibition of the tail growth in 14 days.

Table 1 shows that oestrone in a dose of 0.1 mgm. produced a similar inhibiting growth effect, and 2 mgm. desoxycorticosterone acetate was active to a certain extent in the same direction.

Figures 1 to 3 show the histological changes produced in hypo-

physectomized rats with growth hormone, and with a combination of growth hormone and testosterone.

It can be seen in Figure 1 that the columns of the cartilaginous cells are disorganized and atrophied. Figure 2 shows the effect of 14 daily injections of 5 mgm. growth hormone extract. The cell columns show the characteristic regular arrangement for cartilaginous growth. Figure 3 shows the effects of combined injections of 5 mgm. growth hormone extract and 4 mgm. testosterone propionate. There is no marked disturbance of the regular arrangement of the cartilage cells; there are perhaps slightly less active cells developed in the columns,



Epiphyseal cartilage, tibia of hypophysectomized rats decalcified in 1% phloroglucinol, 5% nitric acid solution, stained with Mallory 120×

FIG. 1. 14 days after hypophysectomy. Untreated.

FIG. 2. 14 days after hypophysectomy. Treated with 5 mgm. growth hormone extract daily.

FIG. 3. 14 days after hypophysectomy. Treated with 5 mgm. growth hormone and 4 mg. testosterone propionate daily.

but no systematic measurements in this direction were carried out. The preponderance of cells over the matrix is perhaps not so marked in this experiment as in the experiment carried out with growth hormone alone. The circulation is obviously increased and so is the calcification.

DISCUSSION

The result of these investigations shows an inhibiting action of testosterone on the cartilage growth activity of the growth hormone. These results were achieved with daily injection of 4 mgm. testosterone propionate. Using a daily dose of 0.05 mgm. to 1.0 mgm. only, Simpson, Marx, Becks & Evans (1944) who measured the width of the tibia of hypophysectomized rats, claimed a synergistic action between testosterone and growth hormone. In table 2 of their paper, however, the combined application of both hormones, shows in series I an inhibition in the growth of the epiphyseal cartilage, and in series II a result which is hardly significant.

A great number of observations have been recorded on the influence of castration on the one hand, and treatment with sex hormone on the other hand, carried out on normal non-hypophysectomized rats, and a certain amount of controversy still exists regarding the experimental results. Thus, H. S. Rubinstein, Kurland & Good-

win (1939) have observed a depressing effect of testosterone propionate on body growths, when injected intraperitoneally in daily doses of 1 mgm., whereas Rubinstein & Solomon (1941) and Rubinstein (1940) showed a stimulating effect of the same hormone in smaller doses (50 mg.) both in normal and castrated young albino rats. The same authors again showed a depressing effect of higher doses in castrated animals. (Rubinstein and Solomon 1941). In all these studies the assumption was made that the effect of testosterone on growth was operated through the pituitary by stimulating depressing, or summing up a growth promoting effect of that hormone on the growth hormone. In the more specific field of cartilage and bone development, the literature is in no better agreement. Silberberg & Silberberg (1941) produce inhibition of cartilaginous growth, increased calcification and accelerated bone ageing, at least, temporarily. Turner, Lachmann & Hellbaum (1940) were unable to detect significant differences in skeletal development of rats, followed up from birth to 87 days, both normal and castrated, treated with testosterone, as compared with normal controls. Clinically, many authors have reported favourable results with the use of testosterone propionate for stimulation of body growth, especially in cases of hypogonadism. Webster & Hoskins (1940); Kenyon, Knowlton, Lotwin, Munson, Johnston & Koch (1942); Finkler, Furst & Cohn (1942) and Goldzieher (1941). All these results were obtained with dosages never exceeding 125 mgm. per week, and in most cases on hypogonadal patients.

As Goldzieher (1941) notes, it may well be that what seems an actual stimulation of growth, is merely the correction by substitution of a lacking balance allowing the restoration of growth. Also McCullagh & McGurl (1940) have reported cases of considerable advance in bone age as judged by epiphyseal closure in relatively short periods, employing high doses during long courses of treatment.

The same effect on growth seen by Zondek (1941) and others, was used by Griffiths & Young (1942) as the basis for an assay method of growth hormone. These authors showed resumption of growth following growth hormone injections, in animals where growth had previously come to a standstill after implantation of 30 mgm. of diethylstilboestrol. It is obvious that the experimental results of these authors are inconclusive regarding peripheral suppression of the action of growth hormone by oestrogen; since the pituitary of their animals was not removed no differentiation of the central and peripheral inhibition can be made on the basis of the data provided by these experiments. The present investigations on hypophysectomized animals, on the other hand, demonstrated conclusively the action of peripheral inhibition, beyond the obvious direct inhibition of the pituitary action by oestrone or testosterone.

Some light may be thrown on the growth/sex hormone correlation, by consideration of the effect of certain endocrinological dis-

turbances on body growth, such as, the eunuchoidal gigantism after prepuberal castration; the cessation of linear bone growth after sexual maturity is reached, when considerable amounts of sex hormones are circulating in the body fluids; the increased linear growth, during puberty, when only small amounts of sex hormones start to enter the body fluids. Other relevant facts are the growth of cartilage that may occur after the climacteric, sometimes even producing acromegalic changes, together with the observation that the growth of male dwarfs can be increased considerably by small doses of testosterone

The influence of sex hormones on the pituitary has been shown in many experiments. The present investigation, however, supports the view that the sex hormones may also directly influence the growth of the bone in the periphery. The experiments described prove an inhibitory action of large doses on the bone growth. A stimulating action, in a sense, as claimed by Simpson et al. (1944) by comparatively small doses, cannot be excluded.

The mechanism of the growth inhibiting action might be based on the increasing circulation rate and increased calcification.

An antagonistic action of small and large doses of sex hormones is quite in conformance with other physiological hormone actions; thus the parathyroid hormone in small doses stimulates the osteoblasts, while large doses stimulate the osteoclasts.

SUMMARY

The daily injection of 4 mgm. testosterone propionate inhibits considerably cartilaginous growth produced by growth hormone, on hypophysectomized rats. The results suggest a peripheral growth inhibitory action of large doses of sex hormone. The bearing of these results on explanations of certain physiologic and pathophysiologic growth phenomena is discussed.

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EFFECT OF FSH AND LH UPON THE OVARIES OF IMMATURE CHICKS AND LOW-PRODUCING HENS

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PRELIMINARY WORK in this laboratory has shown that the injection of FSH into adult laying hens causes the growth of many follicles which may be made to ovulate following intravenous injection of LH. Fraps and Riley (1942) found similar results with PMS pretreatment followed by LH. Injections of gonadotropes, when given in sufficiently high dosages, usually cause cessation of laying within a few days after the first injection (Bates, Lahr and Riddle 1935). Pretreatment with FSH and the administration of LH will permit the ovulation of 2–8 follicles per hen (Nalbandov unpublished) or on the average about 3.5 ovulations per hen (see also Fraps and Riley 1942).

As a follow-up to the studies on the laying hen it was of interest to determine the effect of the FSH-LH combination upon the resting or intermittently functioning ovary of the adult hen. It is also of theoretical and of practical importance to determine whether the inactive ovary of the adult hen is comparable, in its ability to respond to gonadotropes, to the inactive ovary of the immature chick.

Some studies have been reported on the effect of various gonadotropes on the immature chicken ovary. Reexamination of this problem was however thought worth while because of lack of agreement of reported experiments, but mainly because preparations were available to us which were known to be capable of causing ovarian stimulation and ovulation in the sexually mature hen. The general reports in the literature fall into two categories. One group of workers claims that the ovary of immature chickens is incapable of being stimulated with gonadotrophic hormones. Other workers (Domm 1937, Domm and Van Dyke 1933, Asmundsen *et al.* 1935, 1937 and others) agree that female chicks become progressively less sensitive to a given dose of gonadotrophic hormone with increasing age; that in the younger bird primarily interstitial while in older chicks also follicular response may be obtained and that increase in follicular size is usually microscopic in nature and falls far short of approaching ovulatory or even preovulatory size. All workers agree that it was not possible to cause ovulation of follicles of any size in the sexually immature chicken.

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EXPERIMENTAL

A. *Hormones, dosages and length of injections.* The hormones used in these experiments were purified FSH and LH preparations which were kindly furnished by the Difco Company. In a few preliminary trials with laying hens the amounts of FSH and LH were determined which, when injected, would cause cessation of laying, the production of from 4 to 8 follicles of ovulatory size and the ovulation of from 50–100% of the largest follicles. The amounts of hormones necessary to accomplish these effects were arbitrarily called "a hen unit of the FSH-LH combination." In all the experiments described in this report each experimental animal was given a "hen unit" of the two hormones in the sequence described below.

According to labeled potency the amount of FSH and LH equivalent to one "hen unit" turned out to be about 400–500 Rᴜ of FSH (depending on the preparation) and 15 I.U. of LH.

The FSH was slightly contaminated with LH. It caused luteinization of immature rat ovaries when given in dosages of .5 to 5.0 mgs. per rat. The ovaries produced weighed 75–130 mg.

The LH (Luteogen) showed very low activity by the rat seminal vesicle test and seemed to have depressing rather than synergizing effect when given with FSH. In spite of this it was very effective in causing ovulations in chickens.

The LH preparations were singular in another respect. Two of the 3 preparations used in this study were capable of causing oviposition; the third was not. Oviposition occurred in 9 out of 9 hens within 2 to 22 minutes following intravenous injection of LH. Since it was assumed that this effect was due to contamination of the LH with a pressor substance, assays were made by Dr. J. P. Schooley of the Difco Company. Intravenous injection of the LH into an anesthetized dog showed that the LH preparations used were completely free of oxytocic activity. Work on the identification of the active principle in the anterior pituitary causing oviposition is being continued.

In all the experiments with chickens described here the FSH was injected subcutaneously once daily for 12 days. On the 12th day LH was given intravenously and autopsy was performed on the 13th day. Comb measurements (length×height) were taken on the day of the first injection and again at autopsy. The ovaries and oviducts were removed at autopsy and weighed. The diameters of the largest unovulated follicles were determined. The stimulated unovulated follicles which were smooth-walled and well distended were called normal in contrast to those which were puckered. Presence of whole unruptured ova in the body cavity was taken as evidence of ovulation.

B. *Animals used and results.*

1. Experiments with immature chicks. One hundred White Leghorn chicks, all of them hatchmates, were raised in the laboratory under standard conditions. At the ages of 30, 60, 90, 120, 150 and 180

days 16–20 chicks were selected at random. Of these, 8 or 10 were designated as controls while an equal number was injected with one "hen unit" per chick of FSH and LH in the manner described earlier in this paper. Thus the effect of a dosage of hormone known to be capable of stimulating an adult chicken ovary and of causing ovulations was tested on a homogeneous group of immature chickens at various ages up to sexual maturity.

At all age levels hormone treatment caused some increase in ovarian and oviducal weights (Table I). However between the ages of 30 and 150 days these differences are not phenomenal and occasionally

TABLE 1. EFFECT OF FSH AND LH ON IMMATURE FEMALE CHICKENS OF INCREASING AGE

Age at first injection (days)	No. of animals	Treatment	Comb area		Ovary weight (mgs.)	Oviduct weight (mgs.)	No. of follicles over 1 mm. in diameter	Average diameter of follicles
			At first injection (L×H)	At autopsy (L×H)				
30	8	Injected	.18	.50	.09	.02	None	Less than 1 mm.
	8	Control	.18	.19	.02	.01		
60	8	Injected	2.55	4.06	.23	.13	None	Less than 1 mm.
	7	Control	2.41	3.06	.17	.10		
90	8	Injected	4.41	5.35	.23	.14	None	Less than 1 mm.
	8	Control	4.34	4.92	.23	.10		
120	8	Injected	8.31	11.79	.42	1.10	None	Less than 1 mm.
	8	Control	7.93	12.68	.34	.38		
150	8	Injected	21.07	32.03	2.22	9.97	11[1]	1.28
	8	Control	21.82	32.20	2.02	7.94	17	.73
180	10	Injected	45.66	57.68	43.81	39.16	72[2]	3.69
	10	Control	41.37	46.83	19.79	25.99	27[2]	3.90

[1] 4 birds negative.
[2] Figures based on 6 birds in control group and 5 birds of injected group (see text).

not even statistically significant (30 and 90 days). At 30, 60, 90 and 180 days of age injections significantly increased comb size, while at 120 and 150 they did not.

Histologically the effects of FSH from 30 to 120 days were microscopic in magnitude and were restricted to the medullary portion of the ovary. At 120 days one chick (out of 8) had one follicle which measured .95 mm. in diameter. During the first 120 days of life none of the follicles in the ovary, in spite of powerful hormone dosages, was stimulated beyond the development found in the controls. The first apparent effect of the treatment upon the germinal epithelium occurred at 150 days of age. (Table I). At 180 days this situation was still more pronounced in that there were about one-third as many follicles over 1 mm. in diameter among the treated group as there were among the controls. The average diameter of follicles was almost the same in both groups. It must be emphasized that in none of the birds up to 180 days of age was it possible to cause ovulations or to cause the maturation of follicles even approaching ovulatory size.

During the 13-day experimental period (with) birds 180 days of age, 4 of the control group began to lay and performed as follows:

1. Two small eggs laid (days 2 and 7 of experiment) 1 egg in oviduct at death.
2. One small egg laid (day 10)
3. One soft-shelled egg in uterus at death.
4. One ovum in body cavity at death.

In the control group then 6 ovulations in 4 birds had occurred during the 13–day experimental period. During this same period the following observations were made at autopsy in the experimental group.

1. Three ova in body cavity, 1 egg in oviduct.
2. One soft-shelled egg in utero.
3. Four ova in body cavity.
4. Two ova in body cavity, 1 egg in oviduct.
5. Three ova in body cavity.

In the experimental group 15 ovulations were produced but laying did not occur.

Since the injection period of the oldest group of chicks (180 days) inadvertently coincided with the normal commencement of laying in the control group it was of interest to determine how long before laying began normally it would be possible to cause follicular maturation and ovulation. For this purpose an additional series of experiments was conducted. In this second experiment, groups of 25 pullets were injected with one "hen unit" for 13 days beginning at 160 days of age. On the day on which the experiment ended for the first group a second group was started on the injections and so on.

These trials showed that it was possible to force ovulation in a few individuals not more than 2 to 3 weeks before laying would have commenced normally. Of a group of 25 birds 3 were stimulated sufficiently to produce follicles of ovulatory size. One pullet was caused to ovulate 3 ova 22 days before the first bird of the control group laid her first egg. The other 2 pullets were caused to ovulate 8 and 15 days respectively before the first normal ovulation of their controls. The remaining birds showed ovaries in various degrees of stimulation ranging from no stimulation to follicles 1 cm. in diameter.

2. *Experiments with mature, non-laying hens.* Seven hens none of which had laid over 25 eggs during the entire preceding year, were selected from a population of birds which had produced from 150–300 eggs during the same interval. The 7 hens selected may be regarded as extremely low producers or even non-layers. Their ovaries as a rule were small, weighing 2–7 grams and containing many follicles about 1 mm. in diameter. Occasionally and at unpredictable intervals the ovaries flare up, ovulate 1 or 2 eggs, then become quiescent again. The hens selected were kept in laying cages for a period of 60 days before the injections were begun. During this observation period a total of 4 eggs was laid by the 7 hens (Table 2). This represents a rate of production of .0095 eggs per hen per day while a group of normally laying controls laid .71 eggs per hen per day during the same interval.

The hormone treatment had a very pronounced growth-stimulating effect upon the ovary and indirectly, the oviduct, the weight of the ovary being inversely dependent upon the number of ovulations resulting from LH treatment. There was also an appreciable increase in comb size, indicating that not only the gametogenic but also the medullary (androgen secreting) portion of the ovary was stimulated. By comparison, the 4 uninjected controls showed no increase in size of ovary, oviduct or comb (Table 2). In this group of hens, with originally in-active ovaries, a total of 21 ovulations or an average of 4.2 ovulations per hen were produced. One hen (No. 7, Table 2) began to lay on the 4th day after the first injection and laid 5 eggs during the

TABLE 2. EFFECT OF FSH AND LH ON THE OVARIES OF NON-LAYING ADULT HENS

Hen No.	No. of eggs produced in 60 days prior to 1st injection	Comb area		Weight of		Number of		Number, type and average size (mm.) of ovarian follicles
		at 1st injection (cm.²)	at last injection (cm.²)	Ovary (gms.)	Oviduct (gms.)	ovulations (autopsy)	eggs laid during experiment period	
1	2	10.34	12.5	92.0	45.0	2	0	5 normal 33.4
2	0	18.27	22.11	75.5	67.0	5	1[2]	6 normal 30.0
3	0	10.00	11.22	72.0	34.0	4	0	4 normal 22.8
4	2	9.18	15.39	26.0	62.5	5	0	0 normal all less than 1 mm.
5	0	24.48	30.24	155.5	73.5	1	0	10 normal 31.8
6	0			68.0	54.4	4	0	4 atretic
7	0					0	5	(laid days 4, 5, 6, 11, 12)
Average	.0095[1]	14.45	18.29	97.8	67.3	4.2	—	4.1 30.2
4 Control	0	28.38	28.27	5.5	10.9	0	0	0 all small

[1] Eggs per day per hen.
[2] Egg in uterus at autopsy.

13 day experimental period. She had no ovulations at autopsy and it is the only individual which deviated from the performance of the rest of the group.

3. *Experiments with mature low-producing hens.* Another group of 7 hens was selected from the same population as the non-laying group. The average production of this group during the 60-day pre-experimental observation period was .23 eggs per hen per day in contrast to the .0095 eggs laid by the non-laying group and the .71 eggs produced by the normal controls. These seven hens, laying 1 egg in about 4 days, may be classified as belonging to a low-producing group. The effects of hormone injection upon this group are summarized in Table 3. The comb, oviducal and ovarian responses of this group were similar to those of the non-laying group. The significant difference between these 2 types of birds lies in the fact that in the low producing group FSH injections alone initiated laying. Laying began soon after FSH injections started and 4.4 eggs per hen were laid. This represents a rate of lay of .33 eggs per hen per day against .23 eggs per hen per day laid before injections began. Of the 31 eggs laid, 17 were laid from the 3rd to the 7th days of the injection period, 12 from the 8th to the 12th while 2 were recovered from the uterus at autopsy. Twenty-three eggs were normal while 8 were soft-shelled.

In this group one hen (No. 7, Table 3) was refractory but some ovarian stimulation was obtained.

Also of interest is the fact that while the hormone treatment accelerated laying in the low producing group the number of ovulations obtained per hen following LH injection was very much lower than the number of ovulations which were obtained in this experiment with

TABLE 3. EFFECT OF FSH AND LH ON THE OVARIES OF LOW PRODUCING HENS

Hen No.	No. of eggs produced in 60 days prior to first injection	Comb area		Weight of		Number of		Number, type and average size (mm.) of ovarian follicles
		At first injection (cm.²)	At last injection (cm.²)	Ovary (gms.)	Oviduct (gms.)	Ovulations (autopsy)	Eggs laid during experiment period	
1	20+1[2]	13.25	21.78	79.5	52.5	0	5+1[2]	9 normal 27.0
2	14	18.56	24.48	83.5	63.0	0	2+3[2]	5 normal 26.0
3	6	24.79	27.38	72.0	44.5	1	1+1[2]	3 normal 21.7
4	8	39.15	53.35	65.0	25.0	1	3+3[2]	3 normal 22.7
5	35	40.80	49.68	9.5	29.5	2	7	1 normal 0.9
6	0	19.50	24.50	51.9	36.3	3	5	no record
7	11	6.46	6.40	36.0	35.5	0	0	4 normal 18.3
Average	.23[1]	23.22	29.65	56.8	40.9	1	4.4 (.33[1])	4.2 22.8

[1] Eggs per day per hen.
[2] Soft-shelled eggs.

non-laying hens (Tables 2 and 3) and which were obtained with normally laying hens in previous experiments.

DISCUSSION

1. Effects of gonadotrophins in the chick. The ovary of the chicken consists of the gametogenic portion, and the medulla which is made up of connective tissue and interstitial cells. Stimulation of the ovary, as judged by the increase in weight or by microscopic examination, may then be due to the proliferation of either or both of these layers. If stimulation of the gametogenic layer is accomplished, one may obtain an increase in either number or size or both number and size of follicles. If the avian ovary were comparable to the mammalian ovary, it should be possible to produce follicles of ovulatory size in the chicken beginning with about the 3rd month of its life or before. However, the avian egg cannot be considered mature and ready for ovulation until an appropriate amount of yolk has been deposited during ripening and the egg has grown to a relatively enormous size. In view of this the problem presents itself as follows:

a. Is the prepuberal ovary capable of responding to gonadotrophins?

b. If it is, can the yolk-building material be mobilized from the body and deposited in the ovum causing it to increase to ovulatory size?

It is possible that in the chick, unlike the mammal, the ability to respond to gonadotrophins lies in the ability to mobilize and deposit yolk. If this is the case hormones mainly concerned with general metabolism should be injected concurrently with the treatment with gonadotrophic hormones.

Work done by Domm (1937) Domm and Van Dyke (1933) and Asmundsen *et al.* (1935, 1937) agrees with the results obtained in our experiment from which the following general conclusion may be drawn: During the prepuberal period mainly medullary proliferation results while at later ages follicular enlargement also may be caused. This follicular proliferation is of microscopic magnitude and only at 100–120 days of age can macroscopically visible stimulation be obtained. Even then the size of the follicles falls far short of even approaching pre-ovulatory size. These small follicles (up to .95 mm. in diameter) cannot be forced to ovulate even with heavy dosages of LH. In some pullets ovulation could be brought about 15–22 days before their untreated controls began to lay normally. This supports the assumption that in the immature chicken gonatodrophins alone are incapable of bringing about maturation of ova and that probably some of the metabolic hormones which permit mobilization and deposition of yolk materials must play an important role.

2. The different effects of gonadotrophins on adult laying, low producing and non-laying hens may be summarized and explained as follows—

a. In the mature laying hen FSH causes cessation of laying and the maturation of several ova of ovulatory size which are capable of ovulation following administration of LH. It seems plausible that the reason for this response is the fact that there is a graded series of from 2 to 4 follicles of ovulatory or preovulatory size. When exogenous FSH is injected, all of these largest follicles are caused to grow rapidly bringing about hormonal im-balance, which results in non-release or non-effectiveness of the hen's own LH. This leads to cessation of laying.

b. In the low producing hen FSH causes speeding up of laying and results in very few ovulations following LH injection at the end of FSH treatment. In the ovaries of these hens there is no graded series of primary, secondary, tertiary etc. follicles as there is in the normally laying hen. Usually there is only one large follicle, the one which is elected to grow to ovulatory size. Following its ovulation there is a break in the laying activity of several days (in our group of birds on the average 5 days) until the next follicle reaches ovulatory size. If exogenous FSH is injected into such a hen the rate of follicular growth and the reciprocal endocrine relations are caused to approach the conditions existing in the normally laying hen. The fact that very few ovulations were obtained in this group of hens following LH injection, indicates that in this case there was no boosting of follicles *en masse* to ovulatory size. The amounts of FSH injected were sufficient to establish an hierarchy of follicles similar to the graded series found in the normally laying hen, but without causing an endocrine unbalance.

The reason for the high proportion of soft-shelled eggs obtained in this group is not clear.

c. In the non-laying group of hens FSH injection alone did not lead to laying, as it did in the low producing birds. Here it caused the maturation of about 4.2 ova per hen, which may be made to ovulate following LH injection. In such birds the follicles were very small (all less than 1 mm. in diameter) and the injected FSH will act upon many follicles of similar size causing them to grow as a unit, and apparently failing to establish a graded series. Thus many follicles will reach ovulatory size at the same time. It is assumed that this mass of "started" follicles is sufficiently potent endocrinologically to upset the hormonal balance, and thus to prevent the animals own LH from acting. Exogenous LH caused the ovulation of many follicles in this group.

SUMMARY

Female chicks were injected with one "hen unit" of FSH followed by LH at 30, 60, 90, 120, 150 and 180 days of age. Some increase in ovarian and oviducal weights resulted. No follicles of ovulatory size were produced before 180 days of age. It is possible to produce follicles of ovulatory size and cause them to ovulate 8–22 days before their uninjected controls would commence to lay normally. In non-laying hens FSH and LH caused the production and the ovulation of an average of 4.2 ova per hen following injection of LH. One hen of 7 began to lay and laid 5 eggs in 13 days. In low-producing hens FSH and LH treatment caused 6 of 7 hens to commence laying. In this group only 1 ovulation per hen was produced following the LH injection.

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BIOLOGICAL ASSAY OF ADRENAL CORTICOIDS[1]

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THE ORIGINAL RESEARCH of Britton and Silvette in 1932 definitely established the relationship of the adrenal to carbohydrate metabolism. These authors showed that extracts of this gland were capable not only of restoring the blood sugar and liver and muscle glycogen stores in adrenalectomized animals but also would influence carbohydrate metabolism in the normal animal. At that time they suggested that the increase in hepatic glycogen might form the basis of a test for measuring the potency of adrenal extracts. It was not, however, until 1942, that Reinecke and Kendall published a quantitative method for the assay of adrenal hormones based upon the ability of these substances to cause deposition of glycogen in the livers of adrenalectomized fasted rats. More recently, in 1944, Olsen and his associates have evaluated the Reinecke Kendall test and have suggested certain modifications such as the pre-feeding of a high protein diet to increase the total response of the animal to a given dose of adrenal cortical hormone and to improve the linearity of the curve of the logarithm of the dose plotted against the response.

In this clinic, we have been interested for some time in the excretion of corticoids in the urine of normal and pathological cases and under varying conditions of stress, such as trauma, burns and infections.

As the method of Reinecke and Kendall and also that of Olsen and associates was not sensitive enough to detect the very small amounts of corticoids in normal urine, an attempt was made to develop an assay method which would meet this requirement. The mouse was chosen as the test animal and the general outline of the Reinecke Kendall procedure was followed with the introduction of certain modifications.

Long, Katzin and Fry in 1940 reported that the normal fed mouse, when administered adrenal cortical hormone, accumulated unusually large amounts of glycogen in the liver and Dobriner found this also to be true for the fed adrenalectomized mouse. The possibility that the administration of dextrose would improve the

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sensitivity of the assay compared to that in the fasted adrenalectomized mouse was therefore investigated. Various amounts of glucose were given and it was found that 70 mg. could be administered in divided doses without causing any significant increase in the liver glycogen in the control animals. When this amount of glucose was given with adrenal cortical extract, a striking increase in the liver glycogen was observed. As various colonies of mice may differ in their response to glucose, the minimum dose should be ascertained for each colony. Preliminary studies on the development and application of this method have been reported in 1944 and 1945. A test based upon the use of fed adrenalectomized mice has also been developed by Eggleston and Dobriner. (1944 and 1945)

The following procedure for the assay was adopted:

METHOD

Male white mice from our own colony, weighing 20–25 gm. were used. As the response to adrenal cortical extracts may vary with different strains of mice, it is important to keep to the same strain. Two days before adrenalectomy the mice are removed from their stock fare of Purina dog chow and placed on the McCollum diet which contains 26% protein and 52% carbohydrate. The animals are anesthetized with nembutal or ether and are adrenalectomized by the usual lumbar route. Following adrenalectomy they are placed in a constant temperature room or box at 76° C. and are maintained on the McCollum diet, 0.9% NaCl containing 5% glucose is given as drinking water on the first post-operative day. This solution is removed on the morning of the second post-operative day and 0.9% NaCl is substituted throughout the remainder of the test. The giving of glucose immediately following adrenalectomy almost completely eliminates the mortality due to operative shock. On the third post-operative day food is removed at 5:00 P.M. and the mice are fasted until the following morning at which time the drinking water is also removed. On the fourth post-operative day, beginning at 9:15 A.M. a total of seven injections are given at 9:15 A.M., 10:00 A.M., 10:45 A.M., 11:30 A.M., 12:30 P.M., 1:30 P.M. and 2:30 P.M. The material to be tested is taken into solution in 5% glucose and 10% alcohol. 0.20 cc. are given subcutaneously for each injection, so that each mouse receives a total of 1.4 cc. extract containing 70 mg. glucose. At 3:30 P.M. the mice are weighed and anesthetized with sodium amytal (0.2 cc. of a 1.8% solution). The livers are quickly removed and plunged into 4 cc. of hot 30% KOH contained in a 15 cc. graduated centrifuge tube. The tubes are heated in a boiling water bath and frequently shaken until all the tissue is in solution. The glycogen is precipitated by the addition of 1.2 vols of 95% alcohol. The tubes are heated until the mixture just begins to boil, cooled in an ice bath and centrifuged. The supernatant liquid is poured off and the tubes are allowed to drain. The sides of the tubes are washed down with 0.5 cc. alcohol and again allowed to drain. Final traces of alcohol are expelled by heating the tubes for a few minutes in the hot water bath.

The glycogen is hydrolyzed and the glucose is determined by the method of Good, Kramer and Somogyi. The glycogen is expressed in terms of mg. of liver glucose per 100 gm. of mouse body weight.

Standard:

The reference standard is 11-dehydro-17-hydroxy corticosterone (Crystalline Compound E of Kendall). The biological activity equivalent, that of one microgram of 11-dehydro-17-hydroxy corticosterone in terms of amount of glycogen deposited, is defined as one glycogenic unit.

Preparation of Urinary Extracts

For urines containing a normal or low titer of glycogenic activity a complete 48-hour specimen is necessary for the assay; for urines containing a high titer a 24 hour specimen is sufficient. The urine is adjusted to pH 1 with HCl or H_2SO_4 and extracted three to four times with ethylene dichloride: Chloroform may also be used. If any emulsions are formed they can be broken by centrifugation or by allowing the mixture to stand for an hour. The clear ethylene dichloride extract is evaporated almost to dryness under reduced pressure, the temperature of the water bath should not exceed 55° C. The residue is taken up in 30 cc. chloroform and the chloroform is extracted 3 times with 5 cc. of cold N/10 NaOH and 3 times with water. These washings are re-extracted with chloroform. The combined chloroform is evaporated down to a volume of approximately 1–2 cc. and is transferred into a test tube with small amounts of chloroform. The test tube is placed in a water bath at 50° C. and the remainedr of the chloroform is removed under a stream of nitrogen. The dry residue is stored in the cold until ready for assay.

Preparation of Extract for Assay

Six to eight mice must be used for each assay. For normal male urine the equivalent of six hours of urine is administered to each mouse, whereas for normal female urine or urines expected to be low in glycogenic activity, the equivalent of 8 hours of urine is given to each animal.

The following will illustrate the manner in which the residue is prepared for assay so that the final extract will contain 10% alcohol plus the required amount of glucose which for our colony is 70 mg. per mouse. For example, the residue obtained from a 48 hour specimen of urine is to be assayed on eight mice. Each mouse receives seven injections of 0.2 cc. so that the final extract is made up to $7\times0.2\times8=11.2$ cc. and should contain $70\times8=560$ mg. of glucose. A 10% glucose is accurately prepared and 5.6 cc. of this solution are measured into a 15 cc. graduate tube. The urine residue is dissolved in 1.12 cc. of alcohol and this solution is run slowly drop by drop from a pipette into the 10% glucose solution, the tube being shaken continuously during the addition of this material. The original test tube and pipette are rinsed out with water which is added to the extract and the final volume is made up to 11.2 cc. with water. The extracts are stable for 24 to 48 hours at ice box temperatures.

RESULTS

1. *Effect of Added Glucose and Diet upon the Sensitivity of the Assay.* In the search for a suitable assay method for measuring small amounts of cortical adrenal activity, it was thought that if the mouse were substituted for the rat in the Reinecke Kendall test a proportionally smaller amount of adrenal cortical hormone might be required to

cause liver glycogen deposition. However, 0.1 cc. of adrenal cortical extract (Connaught) failed to cause a significant increase in liver glycogen in the fasting mouse. In order to increase the sensitivity of the assay it was decided to administer glucose along with the extract to be assayed. The maximal amount of glucose that could be given to the fasted adrenalectomized mouse without significantly altering the liver glycogen content at the end of assay was determined. The liver of the fasted adrenalectomized mouse is quickly depleted of glycogen. At the beginning of the test, on the fourth post-operative day, following a fast of 16 hours, as listed in Table I, the liver contained an average of 2.1 ± 0.5 mg. glycogen per 100 gm. mouse.[2] Six and a half

TABLE I. EFFECT OF SOLVENT, DIET AND GLUCOSE UPON LIVER GLYCOGEN DEPOSITIONS IN THE ADRENALECTOMIZED MOUSE

Extract administered	Solvent	No. of mice	Glucose per mouse mg.	Diet prior to assay	Mean glycogen deposition mg./100 gm. mouse
None	None	12	None	A (fast 16 hrs.)	2.1 ± 0.5[1]
None	10% Alcohol	12	None	A (fast 22½ hrs.)	1.8 ± 0.5
None	10% Alcohol	34	70	A	4.1 ± 0.5
500 cc. urine	10% Alcohol	9	None	A	5.3 ± 1.0
500 cc. urine	10% Alcohol	9	None	B	6.7 ± 1.0
500 cc. urine	10% Alcohol	15	70	A	87.5 ± 4.3
500 cc. urine	10% Alcohol	9	70	B	86.0 ± 6.4
0.1 cc. adrenal cortical extract (Connaught)	10% Alcohol	9	None	A	2.7 ± 0.4
0.1 cc. adrenal cortical extract (Connaught)	10% Alcohol	9	70	A	37.0 ± 1.0
0.1 cc. adrenal cortical extract (Connaught)	Oil	6	70	A	2.5 ± 0.5
400 cc. urine	Oil	6	70	A	29.6 ± 3.2
400 cc. urine	10% Alcohol	9	70	A	64.9 ± 5.9

[1] Deviations are expressed in terms of the standard error of the mean.

hours later in a group of 12 controls the average glycogen content is 1.8 ± 0.5 mg. per 100 gm. mouse.

Forty, 50, 70, 100, 150 and 200 mg. of glucose were given in 7 divided doses, as outlined in the method. With the administration of 150 mg. and 200 mg. there was a definite increase in deposition of glycogen above the control level, while with 100 mg. the value was on the borderline. Seventy mg. was finally chosen as the amount to be given. In a series of 34 control adrenalectomized mice, given 70 mg. glucose over a period of 5½ hours and killed one hour later, the livers contained an average of 4.1 ± 0.5 mg. glycogen per 100 gm. mouse. There was a striking increase in the response obtained with both adrenal cortical and urinary extracts when 70 mg. of glucose per mouse were added (Table I).

[2] The deviations are expressed throughout in terms of the standard error of the mean.

An amount of 0.1 cc. adrenal cortical extract (Connaught) was not effective in causing liver glycogen deposition in the fasted adrenalectomized mouse. However, when 70 mg. glucose was injected along with the extract a deposition of 37.0 ± 1.0 mg. per 100 gm. mouse was obtained. Similarly when an extract equivalent to 500 cc. of post-operative urine was injected no response was obtained; with added glucose 87.5 ± 4.3 mg. glycogen per 100 gm. mouse was deposited. This increase in sensitivity of the method allows the measurement of small amounts of active substances which would otherwise not be detected.

For 2 days prior to adrenalectomy and throughout the remaining period of assay, the mice were placed on the McCollum lactation diet (Diet A). This diet contained 26% protein and 52% carbohydrate. Following the suggestion of Thayer and his associates that a high protein diet favors a greater deposition of liver glycogen in the rat following the injection of adrenal cortical hormones, a comparison of liver glycogen depositions in mice on the two types of diets, high carbohydrate and high protein was made. The high protein diet OG2 of Olsen et al., was used (Diet B), which contained 58% protein and 12% carbohydrate.

An extract equivalent to 500 cc. urine was administered to 4 groups of mice—2 groups were maintained on the high carbohydrate diet—the other two on the high protein diet. The extract was given to the mice first without added glucose and then with the addition of 70 mg. From the results tabulated in Table I, it will be observed, that when no glucose was administered, there was only a slight increase in liver glycogen over the control value with both diets A and B. However, with the addition of 70 mg. glucose the glycogen depositions for Diets A and B were 87.5 mg. and 86.0 mg. respectively. There was no significant difference in the response of mice on these two diets.

(2) *Effect of Solvent upon the Glycogenic Response in the Mouse.* When the same amount of material was administered in 10% alcohol solution and in oily solution (sesame oil) the amount of glycogen deposited was greater when the material was dissolved in 10% alcohol. This was true for both the adrenal cortical extract and the extract obtained from post-operative urine (Table I).

(3) *Reproducibility of Assays.* An extract obtained from 200 liters of urine collected from patients who had undergone surgical treatment was purified according to a procedure outlined in a previous paper, and was assayed repeatedly at various dose levels over a period of 6 weeks. Six to ten mice were used for each assay. In Fig. 1, are charted the variations that occur in glycogen deposition values in individual mice and the mean values for each assay, and in Table II are listed the mean deposition values for the total number of mice used at each dose level and the statistical analysis of the results. When the mean glycogen depositions were plotted against the logarithm of

the dose, a linear relationship was observed. Although there was a wide range in the response of individual mice the mean value for the separate assays fall within a narrow range, for example, in 4 assays at a dose level of 500 cc. urine whereas the liver glycogen ranged in individual animals from 47 to 147 mg. per 100 gm. mouse; the mean values for groups of eight to nine mice varied only from 83.5 to 88.0. Further the mean glycogen deposition values for each dose level of urinary extract were shown to be significantly different from one another. In each instance the significance factor of Fisher was greater than 2.

TABLE II. LIVER GLYCOGEN DEPOSITIONS OBTAINED WITH ADRENAL CORTICAL STEROIDS AND URINARY EXTRACTS IN THE ADRENALECTOMIZED MOUSE

Compound	Amount	No. of mice	Mean liver glycogen	Significance of differences
	microgram		mg./100 gm. mouse	't'[2]
11-dehydro corticosterone	40	8	42 ± 4.8[1]	—
	80	8	69 ± 7.9	2.9
	160	9	94 ± 7.1	2.6
11-dehydro-17-hydroxy corticosterone	10	8	33 ± 3.2	—
	10	8	35 ± 2.5	—
	20	8	61 ± 2.0	—
	20	8	63 ± 4.4	6.6
	40	8	91 ± 7.5	3.3
	50	8	102 ± 7.8	1.0
	cc.			
Extract of post-operative urine	100	24	24 ± 2.2	—
	160	28	41 ± 2.5	8.2
	250	28	56 ± 2.8	3.9
	500	32	86 ± 4.0	5.8
	1000	20	108 ± 4.9	3.5
	2000	10	135 ± 4.5	4.6

[1] Deviations are expressed in terms of standard error of the mean.
[2] 't' is the significance factor of Fisher. A 't' factor greater than 2 indicates significant differences.

There was a tendency for a wider variation in the individual assays, when carried out at deposition levels near the control base line and also at the very high levels. The range of liver glycogen best suited for accurate assays in the mouse is between 40 and 110 mg. per 100 gm. mouse. Extracts should be so administered as to give glycogen deposition values falling within this range. Another important factor in obtaining accurate assays is the maintenance of an even environmental temperature. If the temperature falls below 70° C., particularly during the fasting period, less glycogen is deposited for a given dose.

This method has now been used in our laboratory over a period of two years and the liver glycogen response of the control mice and of the mice treated with adrenal cortical hormone has been followed at various intervals. During the greater part of the year the results are

reproducible, however, in the summer months from June to September, a change in sensitivity of the mice has been observed. During these months the standard dose of 70 mg. glucose will cause some

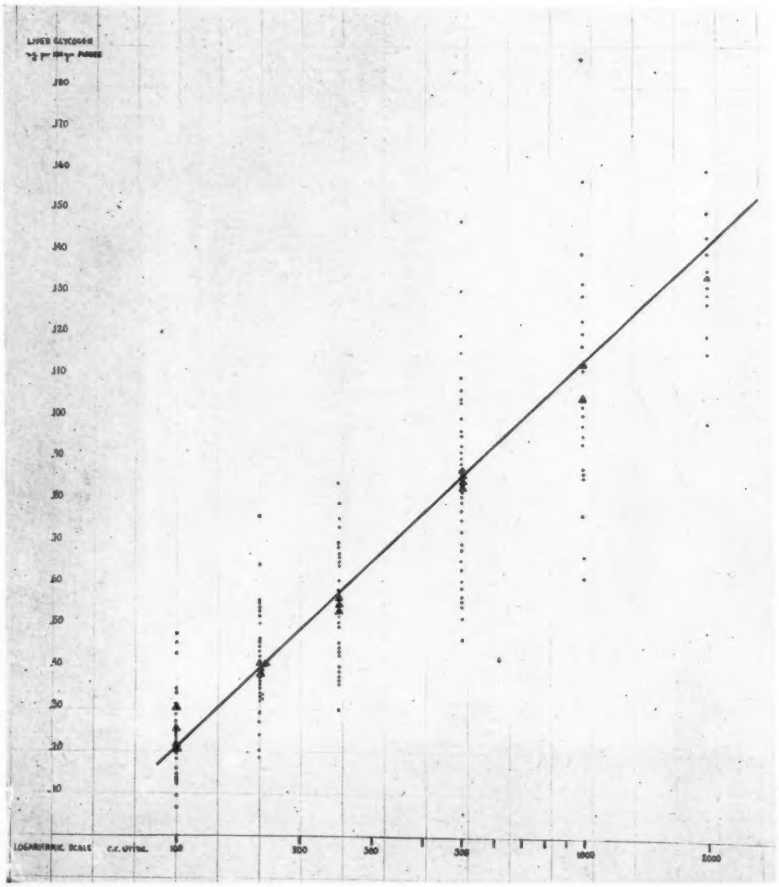

FIG. 1. Logarithm dose-response regression line for urinary extracts. ● Individual mouse liver glycogens. ▲ Mean values for separate assays of groups of 6 to 10 mice.

deposition of glycogen and consequently the deposition values for known amounts of adrenal corticoid are greater. It is possible that this change in sensitivity may not be observed in other laboratories where the climate is less variable, however as a precautionary measure, the sensitivity of the mice should be checked from time to time with crystalline adrenal cortical steroid.

(4) *Glycogenic Activity of 11-dehydro 17-hydroxy corticosterone and*

11-dehydro-corticosterone. The biological activity of these two crystalline adrenal cortical steroids was compared in the adrenalectomized mouse. Under the conditions of our assay, 11-dehydro-17-hybroxy corticosterone was found to be more than 3 times as active as 11-dehydro-corticosterone. The administration of 40 micrograms of 11-dehydro 17-hydroxy corticosterone caused a deposition of 91 mg.,

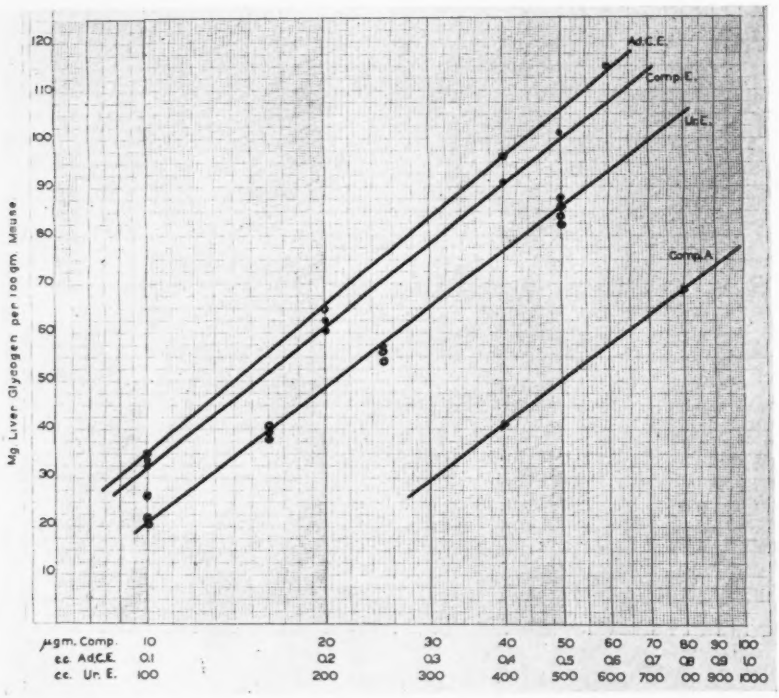

FIG. 2. Logarithm dose response regression lines for adrenal cortical extract, urinary extract, 11-dehydro 17-hydroxy corticosterone and 11-dehydro corticosterone.

whereas it required 100 micrograms of 11-dehydro-corticosterone for a deposition of approximately the same amount of liver glycogen. The glycogen deposition values for various dose levels of these 2 crystalline hormones are listed in Table II. When these deposition values were plotted against the lograrithm of the dose there existed a linear relationship.

In Fig. 2, are charted the various regression lines for 11-dehydro-17-hydroxy corticosterone, 11-dehydro corticosterone, adrenal cortical extract (Connaught Laboratories) and urinary extract obtained from post-operative urine. Although the biological activities of 11-dehydro-17-hydroxy corticosterone (Compound E) and 11-dehydro corti-

costerone (Compound A) are different, the slopes of their regression lines are similar. This is also true for the regression lines of adrenal cortical extract and urinary extract. As these extracts are probably mixtures of various adrenal hormones it is not possible to make any comparison regarding their biological activities. The nature of the active compounds excreted in urine is not yet known.

(5) *Excretion of Urinary Nitrogen.* An attempt was made to determine whether the liver glycogen deposited during the period of assay was derived from the breakdown of protein or was due to a suppression of oxidation of the administered glucose. Urine was collected from groups of 6 mice under varying conditions and the total urinary

TABLE III. EFFECT OF DEHYDROCORTICOSTERONE ON LIVER GLYCOGEN AND URINARY NITROGEN IN FASTED AND GLUCOSE TREATED ADRENALECTOMIZED MICE

No. mice	Treatment	Dehydrocorticosterone administered per mouse	Total urinary nitrogen 12 mice[1]	Total liver glycogen 12 mice[1]
		Microgram	mg.	mg.
12	Fasted	None	39	5
12	Fasted	300	71	12
12	70 mg. glucose per mouse	None	42	10
12	70 mg. glucose per mouse	300	75	210

[1] Collection of urine was made from groups of six mice each, over a period of 6½ hours.

nitrogen and liver glycogen measured. These values are listed in Table III. Fasted and glucose treated mice were given 300 micrograms 11-dehydrocorticosterone. Controls were carried out under the same conditions. This amount of 11-dehydrocorticosterone failed to cause an appreciable deposition of glycogen in the fasted adrenalectomized mice, although there was a definite increase in urinary nitrogen over that of the control mice. When 70 mg. glucose was given per mouse and a total amount of over 200 mg. of liver glycogen was deposited, the increase in urinary nitrogen over that of the controls was of the same order as that observed in the fasted mice, thus suggesting that similar amounts of protein are being catabolized in both groups of mice and also that this amount of glucose does not exert a significant protein sparing action under the conditions of these experiments.

(6) *Hydrolysis of Urine.* Our original work on the extraction of adrenal cortical substances from urine (6) was carried out on unhydrolysed urine. Later it was found that if the urine was acidified to pH 1, an increased amount of activity could be extracted and in most cases the activity is doubled. If after extraction the acidified urine is allowed to stand for 24 hours, small additional amounts of activity can be extracted. A 24 hour specimen of urine was extracted immediately without acidification and was found to contain 98 glycogenic units. The remainder was acidified to pH 1 and re-

extracted with ethylene dichloride. An additional 88 glycogenic units were obtained. On allowing the urine to stand over night at room temperature and extracting a third time with ethylene dichloride, 22 glycogenic units were recovered. However, in carrying out numerous routine assays it has been found inconvenient to allow the urine to stand at room temperature for 24 hours and as outlined in our method, the urine is brought to pH 1, with acid and extracted immediately. The values are therefore slightly lower than those which would be obtained from urine that has been allowed to hydrolyse for 24 hours.

DISCUSSION

The absolute increase in the carbohydrate stores of fasted animals receiving adrenal cortical hormones such as corticosterone or 11-dehydro-17-hydroxy corticosterone has been shown by Long and his associates to occur at the expense of the protein stores of the body. Increase in liver glycogen and blood glucose is accompanied by a parallel rise in urinary nitrogen. In mice receiving a high carbohydrate diet, the relative increase in carbohydrate stores is very much greater, but the increase in urinary nitrogen is much smaller and is in no way sufficient to account for the extra carbohydrate deposited as glycogen. Balance studies showed that the effect of cortical hormones in fed animals was to suppress the oxidation of glucose thus causing an excessive deposition of liver glycogen. In the present method, by the administration of minimal amounts of glucose which have no appreciable effect on liver glycogen in the control animal, it has been possible to increase many times the liver glycogen deposition in the treated animal. The question arises whether under these conditions this increase in sensitivity is due to the inhibition of oxidation of the administered glucose or to the fact that when only the minimal carbohydrate requirement is being supplied then the administration of corticoids would have a gluconeogenic effect rather than a suppression of oxidation of the small amount of available carbohydrate. Preliminary studies on the excretion of urinary nitrogen during the period of assay in the fasted and glucose treated mice, with and without adrenal cortical hormone suggest that the increased amounts of liver glycogen obtained in this method are being derived from protein sources. No effect of previous diet on the sensitivity of the assay method was observed. One interesting difference exists between the findings of Reinecke and Kendall and of Olsen and his associates on the potency of 11-dehydrocorticosterone and 11-dehydro-17-hydroxy corticosterone in the rat and that found in the mouse. In the rat 11-dehydro 17-hydroxy corticosterone is only slightly more active than 11-dehydro corticosterone, whereas in the mouse in the present assay it is more than 3 times as active. This may possibly be due to a species difference. The effect of acid in causing an enhanced activity suggests that at least part of the corticoids present in urine are in a conjugated

form. They are readily hydrolysed at pH 1 at room temperature in contrast to the other known conjugated steroids which require a more drastic treatment.

The findings of a seasonal variation in the sensitivity of mice is of interest. The carbohydrate requirement of the mouse during the summer months appears to be somewhat less than during the remainder of the year, consequently higher liver glycogen depositions are obtained with corticoid hormones at this period.

SUMMARY

A modified Reinecke-Kendall test for the assay of small amounts of corticoids has been developed. This method is based upon the deposition of glycogen in the livers of glucose treated adrenalectomized mice. By the administration of measured amounts of glucose it has been possible to increase the sensitivity of the test many times. In the glucose treated adrenalectomized mouse, 11-dehydro 17-hydroxy corticosterone was found to be more than 3 times as active as 11-dehydro corticosterone. By means of this method it is possible to follow the daily urinary excretion of corticoids in the human being.

ACKNOWLEDGEMENT

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HISTOCHEMICAL AGE-CHANGES IN NORMAL AND PATHOLOGICAL PLACENTAL VILLI (HYDATIDIFORM MOLE, ECLAMPSIA)

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IN TWO PREVIOUS PAPERS Dempsey and Wislocki ('44, '45) presented observations on the occurrence and significance of lipoids, glycogen, iron, alkaline phosphatase and basophilia in the human placenta. Since these papers were published, we have accumulated further histochemical data on the normal placenta and also some cytological and histochemical information on hydatidiform moles and on placentas from two cases of severe preeclampsia and eclampsia.

We shall describe for the first time the occurrence of placental acid phosphatase, submitting data regarding the related occurrence of calcium, iron and phosphatases in normal chorionic villi. Second, we shall describe the cytology and histochemistry of two hydatidiform moles which we have examined and discuss the significance of the findings. Third, we shall present an account of the pathological picture of two eclamptic placentas as revealed by cytological and histochemical methods. Finally, we shall comment upon the significance of histochemical age-changes as seen in normal and pathological villi.

METHODS AND MATERIAL

The methods for lipoids, glycogen, iron, alkaline phosphatase and basophilia are the same as the procedures used before (Dempsey and Wislocki, '44, '45), except for the addition of several new features. For the demonstration of glycogen, besides the Bauer-Feulgen stain, we have utilized a silver method devised by Mitchell and Wislocki ('44). Since the previous studies were completed, we have applied Bodian's protargol reaction to the placenta and have already reported upon the occurrence and significance of this reaction in the endometrium and placentas of a variety of animals. From the fact that the reaction is abolished after previous treatment of the sections with acid, we have concluded that the material revealed in the placenta by the Bodian method consists of formed calcium (Wislocki and Dempsey, '45).

For acid phosphatase, Gomori's ('41) method, as modified by Wolf, Kabat and Newman ('43), was employed. Sections of tissue which had been

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fixed in cold absolute acetone were incubated for 24 and 48 hours in a glycerol phosphate solution at pH 4.7.

The material utilized in the present study consisted of ten normal placentas ranging in age from six weeks up to term. In addition to these, two hydatidiform moles and the placentas from two cases diagnosed respectively as severe preeclampsia and eclampsia were studied.[2]

THE DISTRIBUTION OF PLACENTAL ACID PHOSPHATASE

We have examined six normal placentas for acid phosphatase, one each aged 6, 10 and 14 weeks and 4 months and two specimens, at term. Acid phosphatase occurs in the chorionic villi at two periods of pregnancy; during the early part of gestation traces of it are present in the stroma of a few of the chorionic villi while none is found in the trophoblast, whereas late in gestation it has disappeared from the stroma of the villi but has accumulated in considerable quantity in nearly all of the syncytial trophoblast.

The amount of acid phosphatase displayed in the placenta depends upon the length of incubation of the sections in preparing them. The following account is based on the examination of sections which were incubated for 48 hours; a set of sections exposed similarly for only 24 hours showed very appreciably less phosphatase.

In the specimen of 6 weeks no acid phosphatase whatsoever was encountered. In the 10 weeks' specimen traces of phosphatase put in an appearance and by 14 weeks small amounts had accumulated. Acid phosphatase was confined exclusively to the stroma of a few of the chorionic villi where it occurred in patches in the extreme outer part of the stroma just beneath the epithelium covering the villus (fig. 4). In a specimen of 4 months, acid phosphatase had diminished to the merest traces in the stroma of a few of the villi but had not appeared in any other location. In two specimens at term, finally, no trace of it whatsoever was discoverable in the chorionic stroma, but it had meanwhile appeared in the syncytium clothing the villi. The nuclei of the syncytium gave a marked reaction for acid phosphatase (fig. 5), but it occurred also, although in lesser and varying amounts, in the cytoplasm of the syncytium. None was seen at any period of gestation in the cell columns or cell islands, nor in the junctional zone or in the decidua.

It is of interest to compare briefly the distribution of acid phosphatase with that of alkaline phosphatase. In a previous study (Dempsey and Wislocki, '45) the latter was described as occurring in the syncytium in steadily increasing amounts through a considerable portion of gestation up to term. At 8 weeks it was present in the brush border and extreme margin of the syncytium of some of the chorionic

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[2] The specimens used in the present study were obtained through the generosity of Drs. Frederic C. Irving, Duncan Reid, and Arthur Hertig from the Boston Lying-In Hospital.

villi but by 16 weeks had increased to occupy more of the syncytial cytoplasm and to include more villi. At term it had become extremely abundant, occupying nearly all of the syncytium. Moreover it occupied the entire syncytial protoplasm being condensed especially at the outer and inner surfaces of the syncytium and being least evident in the intermediate nuclear zone. None occurred in the nuclei. The nuclei and the immediately surrounding cytoplasm, it will be recalled, are definitely preempted at term by acid phosphatase, and thus it will be observed that the acid and alkaline phosphatases are differently localized within the syncytium.

It follows from the above observations that alkaline phosphatase has a much stronger predilection for the syncytium than acid phosphatase and for a much longer period. The early appearance and relative intensity of alkaline phosphatase in the syncytium led us in our initial report on this topic (Dempsey and Wislocki, '45) to overlook the circumstance that in the middle span of gestation alkaline phosphatase occurs in small amounts in the stroma of occasional chorionic villi just beneath the epithelium, being similar to acid phosphatase in this respect. We now wish to supply this correction and extension of our previous description (fig. 3).

RELATIONSHIPS OF IRON, CALCIUM AND PHOSPHATASE IN NORMAL CHORIONIC VILLI

In the previous section we described the subepithelial localization of small patches and both acid and alkaline phosphatases during the second and third quarters of pregnancy (figs. 3 and 4) and noted the disappearance of the enzymes from beneath the epithelium by the end of the third quarter.

During the second and third quarters of pregnancy, iron and calcium occur in considerable quantities in the stroma of the chorionic villi. These substances are demonstrable just beneath the chorionic epithelium and from these subepithelial deposits granules may be traced into the interior of the villi. Figures 1 and 2 illustrate iron and

Explanation of Plate 1

Fig. 1. A chorionic villus of a normal human placenta of 14 weeks' gestation stained for iron by the Turnbull blue method. The iron is concentrated in the outermost part of the stroma just beneath the trophoblast. Some particles staining for iron extend into the interior of the villus. ×250.

Fig. 2. Parts of three chorionic villi of a normal human placenta of 14 weeks, showing the presence of calcium as revealed by the Bodian protargol method. The calcium occurs in the outermost part of the stroma of the villi in exactly the same distribution as iron in the previous figure. Granules of silvered material extend into the interior of the villi. ×250.

Fig. 3. A chorionic villus from the same placenta, illustrating the very occasional presence of alkaline phosphatase in the stroma of a villus. The phosphatase occurs in the same location as iron and calcium. The small, dark object to the right is a tag of syncytium containing a dense accumulation of alkaline phosphatase. ×250.

Fig. 4. Acid phosphatase seen in traces in the same placenta in the same location in the stroma as iron, calcium and alkaline phosphatase. ×250.

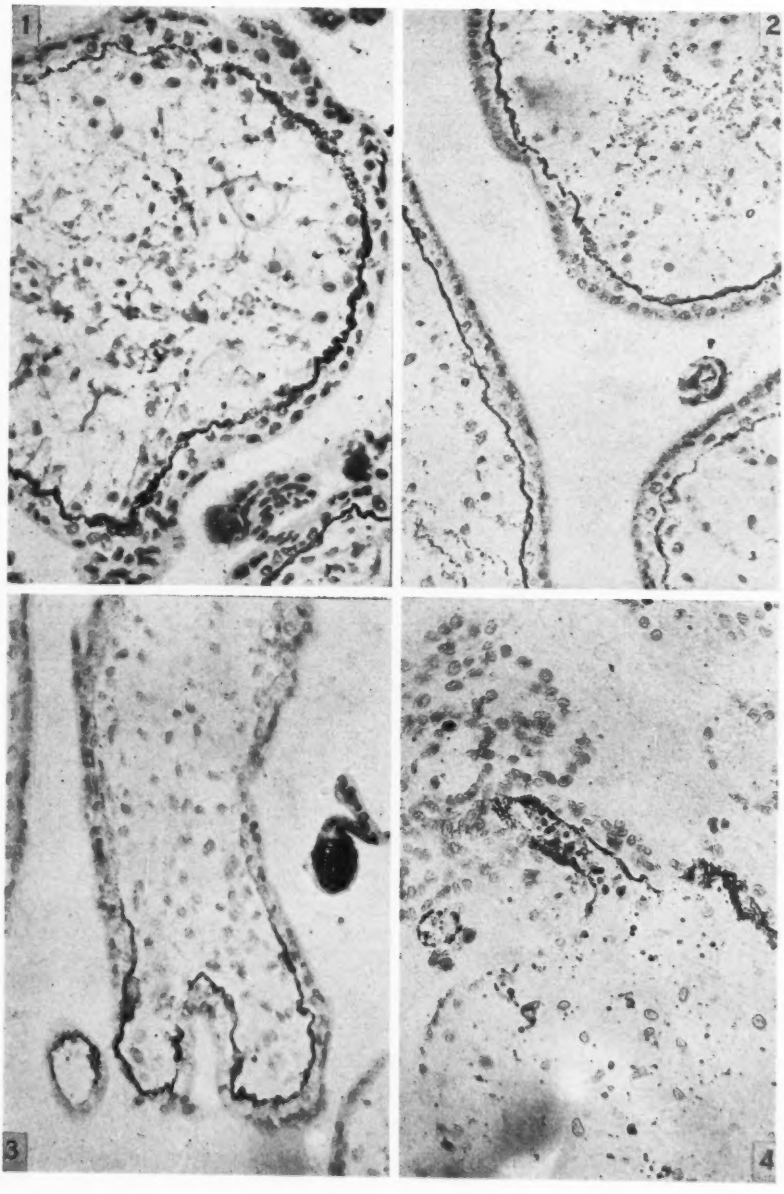

PLATE 1

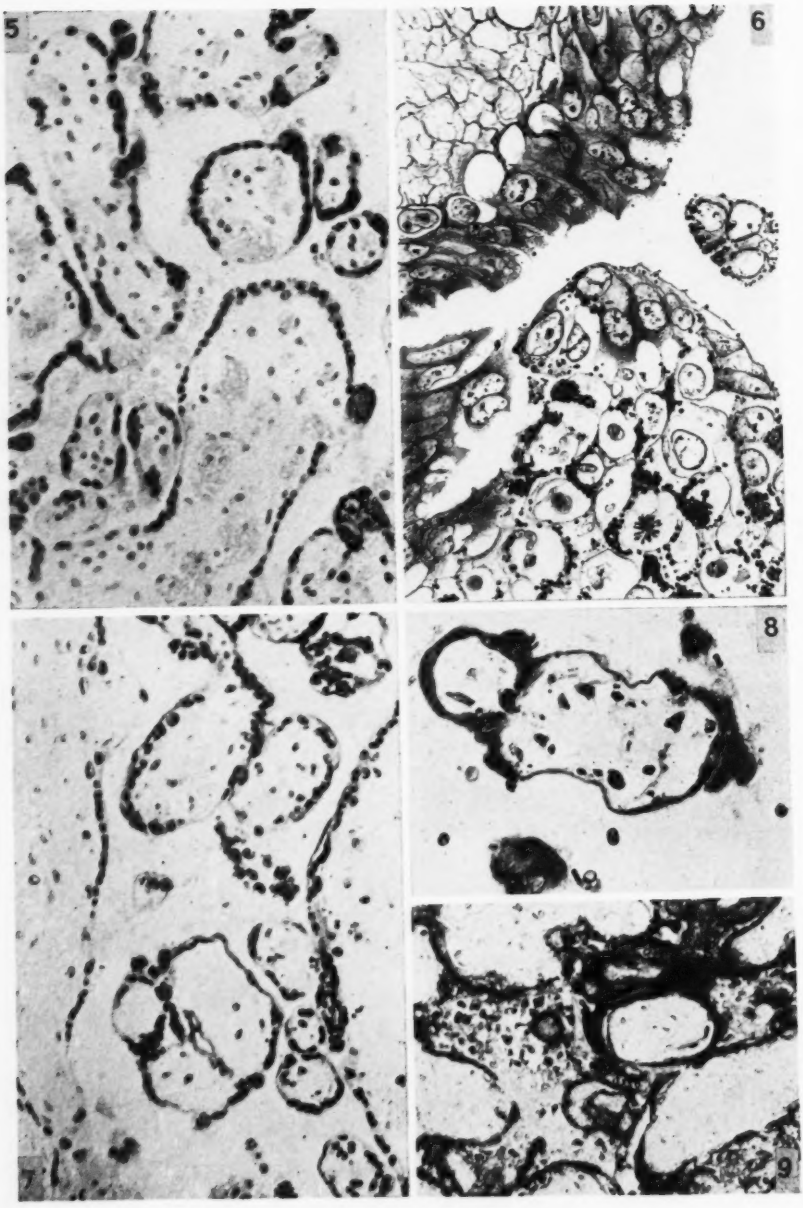

PLATE 2

calcium, as revealed by the Turnbull blue and Bodian protargol reactions respectively, in chorionic villi at 17 weeks of gestation. These deposits diminish as pregnancy advances so that in the last quarter little iron and calcium are normally demonstrable histologically in the chorionic stroma. Both iron and calcium are somewhat variable and patchy in their occurrence in the villi.

The distribution of iron in the stroma of the villi and its diminution in the second half of pregnancy were observed by Hofbauer ('04) in his pioneer investigations of the human placenta. Zancla ('12) confirmed Hofbauer's observations in respect to iron, although Fornero ('12) claimed to find histological iron in increasing amounts in the villi as pregnancy advanced. Both Hofbauer and Zancla remarked upon the somewhat patchy distribution of the iron.

Schönig ('29) reported upon calcium in patchy distribution in the stroma of the chorionic villi and observed its disappearance by the seventh month. He maintained that the subepithelial calcium deposits occurred in the youngest or terminal villi which possessed a two-layered epithelial covering (syncytium and Langhans cells) and in which the blood supply was not yet fully established. Thus he related the accumulation of calcium in certain villi to an insufficient and incompletely developed circulation. Calcium in subepithelial positions is also revealed by the Bodian protargol reaction as utilized in the present study (fig. 2).

The present investigation adds alkaline and acid phosphatases, albeit in very much lesser amounts than either iron or calcium, to the list of substances occurring in the stroma of the chorionic villi.

The relationship of the above observations to placental function is not too readily understandable. We thought at first that the areas exhibiting interstitial deposits of calcium, iron and phosphatase might foreshadow age-changes in the villi. Yet this seemed entirely dis-

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EXPLANATION OF PLATE 2

FIG. 5. Chorionic villi in a normal human placenta at term, illustrating the pronounced reaction for acid phosphatase in the nuclei of the syncytium. A fainter reaction is also visible in the cytoplasm around the nuclei. No reaction is visible in the thinned out, hyaline epithelial plates several of which are visible in the photograph. ×250.

FIG. 6. Drawing of a portion of an hydatidiform mole illustrating typically proliferating cytotrophoblastic cells containing large quantities of glycogen (black). The small isolated mass of syncytium (middle right) contains an abnormal amount of glycogen. Silver method of Mitchell and Wislocki. ×10 ocular, ×40 objective.

FIG. 7. Chorionic villi from a case of severe preeclampsia at 4½ months, illustrating the abnormal accumulation of acid phosphatase in the syncytium, especially in the nuclei. The hyaline epithelial plates, several of which are visible, contain no phosphatase. ×250.

FIG. 8. A chorionic villus from the same case of preeclampsia illustrating the premature ageing of the placenta. Zenker acetic fixation. Eosin-methylene blue stain. The villus shows several extensive thinned out areas of syncytium which are anuclear and have lost their affinity for basic stains. These hyaline areas are far more numerous and extensive than is normal for a placenta of 4½ months. ×350.

FIG. 9. An infarcted area in the placenta from the preeclamptic patient, showing the abnormal density of alkaline phosphatase for a placenta of 4½ months. ×280.

proved as soon as we learned that these substances were destined to diminish with advancing gestation instead of increasing in amount. Thus we were obliged to attach a physiological rather than a pathological significance to the areas in question, and we have come to regard them as areas of concentration of iron and calcium which are being absorbed by the chorion. Why, on the other hand, they should ultimately decrease, although the needs of the growing fetus for iron and calcium presumably increase steadily we have no simple way of explaining. It should be pointed out in this connection that in the deepest layer of the chorion of the sow during the middle span of gestation there is a very conspicuous depot of interstitial calcium and phosphatase which disappears completely in the latter part of pregnancy (Höpping, '37; Wislocki and Dempsey, '46).

We are dealing here probably with depots of substances of a physiological order which are to be distinguished from other deposits of calcium and iron which arise in the ageing placenta and reach their maximum at term. To the latter category belong interstitial infiltrations of iron and formed calcium occurring irregularly in regressing villi, infarcts and cell islands as well as in necrotic foci of the junctional zone. These deposits are frequently associated with the deposition of fibrin.

Two of the most interesting questions in connection with the present findings are why these substances show a patchy localization in the villi instead of being uniformly spread, and in what segments of the system of villi—if there is any constancy in the matter—the patches are located. Their uneven representation suggests that the villi may not be equal in potential throughout their extent, although histologically their structure does not seem to differ very materially from one area to another. Although not much is known about it, placental villi must be composed of thicker primary or stem portions, secondary or intermediate pieces and tertiary or terminal segments. Of the histological differentiation of such possible segments, the most we can say is that the cytotrophoblast and syncytium would appear to be more active as far as growth, penetration and profusion are concerned in the peripheral or terminal regions of the system. Höpping has stated that in his estimation the subepithelial calcium was located in the terminal villi. In our own preparations we are by no means sure as to what regions of the villi contain most of the subepithelial calcium, iron and phosphatase, but from the relative sizes of the villi concerned we have gained the impression that the bulk of the substances occurs in the intermediate region of the villi and not in the terminal ends. This will be appreciated by examining figures 1 and 2.

Höpping suggests further that at the period of gestation concerned, the calcium deposition is contingent on the fact that the fetal vascular tree in the terminal villi is incomplete. There might well be a

relationship between the observed deposition of the various substances which we are considering and circulatory conditions in the villi, but we have at present no real information on this point to guide us. A careful anatomical study of the blood vessels in placental villi in the second or third month of pregnancy might be rewarding in this regard.

THE CYTOLOGY AND HISTOCHEMISTRY OF HYDATIDIFORM MOLES

We have studied two hydatidiform moles by a variety of cytological and histochemical techniques. In the interpretation of our findings we were helped materially by our previous familiarity with the cytology and histochemistry of normal chorionic villi (Wislocki and Bennett, '43; Dempsey and Wislocki, '44, '45).

Since it is well known, we shall not spend much time describing the ordinary structure and histological topography of hydatidiform moles (cf. Hertig and Edmunds, '40). Suffice it to say that the histology of the two specimens which we examined appeared to be fairly typical of the condition. There were numerous villi showing different degrees of transformation into hydatid vesicles. The surfaces of the villi were clothed by trophoblast showing various arrangements. In places the trophoblast was double-layered and moderately normal looking; in other areas it was markedly atrophic and hyalinized, and in still other localities it had undergone marked proliferation, resulting in the formation of small masses of trophoblastic cells variously associated with syncytium. Since there is no need to review the ordinary histology of these different manifestations of regressing and proliferating trophoblast, we shall proceed to the individual cytological and histochemical features which we have investigated.

Cytoplasmic basophilia. It has been established for a variety of cells of the body that cytoplasmic basophilia is related to the presence of ribonucleoproteins (Caspersson, '41). Moreover, it has been suggested that the nucleoproteins play an important role in protein synthesis in the body. Elsewhere we have described the affinity of certain regions of the cellular and syncytial trophoblast in the normal placenta for basic dyes, and have shown that the basophilic substance in these cells is digested by ribonuclease (Dempsey and Wislocki, '45).

In the two hydatidiform moles which we have examined cytoplasmic basophilia, as revealed by staining with eosin-methylene blue, is abundantly present in the trophoblastic cells which have proliferated to form masses and islets of trophoblast. In many places the cytotrophoblastic cells contain cytoplasmic bodies which stain deeply with methylene blue and bear a resemblance to the basophilic Nissl bodies of nerve cells (cf. Dempsey and Wislocki, '45). Moreover, much of the syncytium associated with these proliferating masses also shows an intense stippling or wash of basophilic material, especially pronounced in the perinuclear regions. In places where the

epithelium covering the villi has not proliferated to any extent, basophilia is less intense and in regions where regression or atrophy of the trophoblast has occurred there is no basophilia whatsoever, the cytoplasm having become entirely acidophilic.

Alkaline phosphatase. This substance differs in the two specimens. In the one the merest traces of this enzyme are present in some of the syncytial trophoblast. The second mole contains appreciably more alkaline phosphatase in the syncytium in the various regions where proliferation has occurred, so that some bits of syncytium are quite richly supplied while others are negative. The phosphatase in the latter specimen corresponds in amount and intensity, as far as comparison can be made, to a four months old normal placenta (Dempsey and Wislocki, '45), while the one showing mere traces should be correspondingly younger.

Acid phosphatase. This enzyme was not encountered in either specimen. It should be recalled from a previous section of the present paper that acid phosphatase appears normally in the syncytium at the very end of gestation, whereas, as mentioned above, the moles bear a resemblance to younger placental stages.

Lipoids. Fat droplets are abundantly present in some parts of the syncytium of the two moles. This is the first of the substances so far mentioned which has been seen by previous observers. Bondi ('11) reported that histological fat in the syncytium in hydatidiform moles was normal in amount and distribution, while Ballerini ('12) stated that visible lipoids were increased in the invasive syncytium although they are absent in the syncytium directly covering the vesicles. Our experience with fat droplets stainable by Sudan III has been similar. In the healthy syncytium, on the outskirts of the proliferating cellular masses, fat droplets are numerous. The droplets are nearly uniform in size and relatively large, corresponding in these respects to fat droplets observable in the second and third months of gestation in the normal placenta (Wislocki and Bennett, '43). In the less active syncytium, covering the vesicularly transformed stroma, fat droplets are fewer, while in areas where the syncytium has thinned out and has undergone "hyaline" degeneration no fat droplets at all are visible.

We have examined fresh, unstained sections of the moles under the polarizing microscope and have ascertained that the fat droplets present in the syncytium are birefringent. In this respect the lipoidal inclusions are identical with those which occur in the syncytium of the normal placenta. The trophoblast gives also a positive reaction with Schiff's plasmal reagent and exhibits a greenish fluorescence when examined by ultraviolet light. All of these reactions are also demonstrable in the syncytium of the normal placenta and are indicative of the presence of steroid hormones (Dempsey and Wislocki, '44).

Glycogen. This substance is very definitely increased in the two

moles over its occurrence in normal placental villi. Glycogen is present in abnormal quantities in many of the Langhans cells clothing the vesicular villi as well as in a considerable number of the cells of the proliferating and invasive trophoblastic masses (fig. 6). In the syncytium, also, glycogen is visible in more than normal amounts. Least glycogen is to be found in the hyalinized areas of trophoblast enclosing many of the villi. It should be recalled for comparison that, in the normal placenta, glycogen is limited almost exclusively to the cytotrophoblastic elements of the cell columns and cell islands and that none is present in the Langhans cells and only very occasional traces in the syncytium. In so far as the stroma of the hydatidiform villi has remained cellular, the fibroblasts contain detectable glycogen, whereas in the stroma of the villi which have become acellular no glycogen is encountered. Glycogen is present in ample amounts in the epithelial cells of the uterine glands and in the decidual cells surrounding the mole, being in this respect similar to the normal gravid endometrium.

Iron. The moles are almost free of iron. After application of the Turnbull blue reaction, there is no evidence of the presence of iron in either the cytotrophoblast or the syncytium. There is, however, a faint bluish cast in the interior of many of the villi associated with flocculent material which arises there as the stroma of the villi degenerates. The uterine glands of the decidua in the neighborhood of the molar tissue contain iron as they do also in normal pregnancy.

The Bodian protargol reaction. This reaction, as has been explained in an earlier part of the present paper, reveals subepithelial deposits of calcium in the stroma of the majority of the chorionic villi during the earlier months of pregnancy. In the villi of the two hydatidiform moles which we have examined, on the contrary, the Bodian reaction is completely negative.

Collagen. It is well known that the stroma of the hydatidiform villi very slowly degenerates and eventually liquifies, so leading to the formation of the typical hydatidiform vesicles or cysts.

In our own two specimens one can observe the gradual disappearance of the stromal cells and the degeneration of the collagenous fibers. After applying, the triacid azan stain which colors the collagen of normal chorionic villi distinctly blue, the fibers in the hydatidiform villi are pale and indistinct. When the abnormal villi are silvered by Pap's method for reticulum, the collagenous fibers and fibrils are found to be very distinctly modified. The individual fibers are fragmented, fuzzy in appearance and brownish, being quite unlike normal fibers which are jet black and hair-sharp in outline.

Comments on the histochemistry of hydatidiform moles. Upon reviewing our observations, it will be observed that the trophoblast of hydatidiform moles resembles normal trophoblast in some respects but differs in others.

Similar to normal villi, some portions of the trophoblast of

hydatidiform villi contain appreciable amounts of basophilic substance, some alkaline phosphatase and an abundance of lipoidal droplets which are birefringent and are associated with a positive Schiff reaction.

The trophoblast of hydatidiform villi contains a great excess of glycogen over normal villi. Furthermore, unlike the normal placenta, the molar villi contain no histological iron, give a negative Bodian protargol reaction for calcium, and possess no acid phosphatase.

A comparison of all of these substances in normal and hydatidiform chorionic villi enables us to draw a number of conclusions regarding their significance in the placenta.

1. In previous studies we concluded that the placental steroid hormones are produced in the syncytium (Wislocki and Bennett, '43; Dempsey and Wislocki, '44), basing our conclusion on the presence of birefringent lipoidal droplets in the syncytium which were associated with reactions with phenylhydrazine and Schiff's reagent and with a greenish fluorescence. Birefringent fat droplets associated with the Schiff reaction and greenish fluorescence are also encountered in the syncytium of hydatidiform moles indicating that these pathological growths also produce steroid hormones. In keeping with this conclusion is the observation made repeatedly that the urinary excretion of steroid hormones is frequently increased in this disease.

2. In the normal human placenta we have observed an inverse relationship between basophilia of the syncytium and alkaline phosphatase. The former is abundant in the first half of gestation but declines in the last half of pregnancy as phosphatase accumulates (Dempsey and Wislocki, '45). We also adduced evidence indicating that the basophilic substance may be related to the production of chorionic gonadotropic hormone.

In both of the hydatidiform moles which we have studied moderate amounts of basophilic substance were seen in the more viable parts of the mole. In one of them, only the merest traces of alkaline phosphatase were encountered, whereas in the other specimen somewhat more phosphatase was present. These findings suggest that the first is the younger of the two specimens, since phosphatase increases with age. The variable degree of proliferation present in a given mole and the variable amount of basophilia associated with it probably account for the fact that the excretion of chorionic gonadotropin is subject to wide variation in this disease (Hamburger, '43).

3. The excessive storage of glycogen in the trophoblast of the moles suggests an impairment of the circulation supplying the villi. Dempsey and Wislocki in a previous paper ('44) adduced evidence indicating that glycogen is stored in certain localities in the uterus, placenta and fetus as the result of avascularity, ischemia or anoxemia operating individually or in combination. It is agreed by all writers that, as far as the fetal circulation is concerned, hydatidiform villi are

avascular or are in process of losing their capillaries (Hertig and Edmunds, '40). This impairment of the fetal circulation is probably sufficient in itself to account for the degeneration of the villi and might suffice also to cause an anoxemia followed by glycogen storage. But it seems very likely, although nothing definite appears to be known regarding it, that the maternal circulation bathing the mole may also be deficient. Disarranged anatomically as the mole is, in contrast to the normal placenta, it would appear probable that the maternal blood supply would also be much impaired. Thus deficiences in both the fetal and maternal circulations would seem to be responsible for the regressive changes in the villi and for the accumulation of glycogen.

4. The histology, cytology and histochemistry of the trophoblast of hydatidiform villi all suggest that different areas or parts of the mole are simultaneously in different functional states. Some areas give indications of rapid proliferation (mitoses), other areas show signs of marked functional activity (basophilia, phosphatase, lipoid droplets, still others seem quiescent (glycogen storage), while still further regions are totally inactive and obsolescent (hyaline degeneration).

The histochemical reactions which we have explored supply a certain degree of information regarding the functional state of the viable parts of the molar trophoblast. Those activities which concern placental transmission appear to be definitely impaired (absence of calcium and iron in the villi), whereas those that are concerned with synthesis and secretion are variably retained. The latter involve the birefringent fat droplets and associated reactions and their role in the production of steroid hormones, as well as the basophilic substance and its participation in protein synthesis and the possible liberation of chorionic gonadotropin. Finally, there is glycogen which accumulates as the aerobic mechanism for oxygen utilization in the trophoblast breaks down.

CYTOLOGICAL AND HISTOCHEMICAL EVIDENCE OF PREMATURE AGEING OF THE PLACENTA IN ECLAMPSIA

We have had an opportunity so far to examine only two eclamptic placentas. Nevertheless, this condition is characterized by definite histochemical and cytological lesions which are brought out by the procedures and methods which we have employed. Briefly it maybe said that the placentas in these two cases revealed a premature ageing of the chorionic villi, characterized by the decline of ribonucleoproteins (cytoplasmic basophilia) and the premature accumulation of alkaline and acid phosphatases in the syncytial trophoblast. These histochemical changes are accompanied by "hyaline" degeneration of the syncytium as revealed by ordinary histological stains.

Tenney ('36) and Tenney and Parker ('40), investigating the pla-

centa in toxemias, preeclampsia and eclampsia by ordinary histological means, have stressed that the essential lesion in these conditions consists of a premature ageing of the placental villi. The lesion, according to them, involves an abnormal degree of hyaline degeneration of the syncytium clothing the terminal villi, differing in degree only from hyaline changes occurring normally in the villi of ageing placentas.

Our own studies of ageing placentas and our exploration of eclamptic villi by histochemical means bear out Tenney and Parker's thesis that the placental lesion of eclampsia is related to age-changes in the normal placenta. In the event that this assumption is correct, the cytological and histochemical manifestations of ageing which occur in normal placental villi become extremely important in seeking to define the pathology of the toxemias and eclampsia. In view of this we shall recapitulate briefly the cytology and histochemistry of normal, ageing chorionic villi before presenting our detailed observations and comments on eclamptic villi.

The Histology and Cytology of Ageing Placental Villi. Human terminal chorionic villi become increasingly branched, longer and more slender as gestation advances. The collagen within the stroma of the villi becomes progressively denser and coarser. The trophoblast becomes reduced from a two-layered epithelium to a single-layered syncytium with only very occasional Langhans cells associated with its inner surface. The syncytium clothing the villi becomes reduced in thickness and its specialized border (brush and cytoplasmic streamers) becomes simplified with age (cf. Wislocki and Bennett, '43). In the last third of gestation the sinusoidal capillaries come into closer and closer relationship with the overlying syncytium so that the trophoblast becomes thinned out over the bulging blood vessels. This leads to the formation between the capillaries and the intervillous space of thinned out membranous parts of the trophoblast designated as epithelial plates by Bremer ('16). It has been postulated that these thin membranous areas represent the sites of most ready and easy transfer of substances between the maternal and fetal circulations and vice versa. Bremer himself regarded them solely as excretory in function. These plates increase in number as the placenta ages. In the areas between the plates the syncytial trophoblast remains thicker and the nuclei tend to segregate there, whereas in the plates themselves only occasional isolated nuclei are encountered. We have observed that the individual nuclei aggregated in clumps in the thicker areas of syncytium possess more chromatin and stain more deeply with basic dyes than the more isolated nuclei in the intermediate, thinner areas. In the latter, the chromatin is fainter and oftentime has so little affinity for the basic dye that they have all but faded out of vision. This indicates a gradual loss on the part of the nuclei of their nucleoproteins preparatory to the complete loss of the nuclei themselves.

The syncytial plates, being relatively anuclear and quite homogeneous in appearance by ordinary histological methods, and evincing, besides, an affinity for acid stains, possess the characteristics referred to as "hyaline" in the literature of pathology.

The Histochemistry of Ageing Placental Villi. Fat droplets occur in the syncytium throughout gestation, but diminish perceptibly in size and number by the end of pregnancy. At term the droplets have almost completely disappeared from the epithelial plates. Glycogen is never present in the syncytium except in occasional traces, and none is present in the syncytium at term. Mitochondria (phospholipids) are abundantly present in the syncytium early in pregnancy but decline steadily in number as gestation advances, and relatively few are still present in the syncytium at term (Cf. Wislocki and Bennett, '43).

Basophilia of the cytoplasm is very pronounced in the syncytium early in pregnancy but diminishes steadily with advancing pregnancy. It tends to cling to the cytoplasm around the clumps of nuclei in the syncytium but it disappears relatively early and completely from the syncytial plates. This cytoplasmic basophilic substance in the syncytium must be attributed to ribonucleoproteins (Dempsey and Wislocki, '45). As the basophilia disappears, alkaline phosphatase makes its appearance in the syncytium and increases steadily with ageing of the placenta to reach its peak at term.

The nuclei of the syncytium are small, shrunken and deeply stained by basic dyes until the last month before term when some of them begin to lose their basophilia. This loss occurs mainly in the more isolated single nuclei and extends very slowly to involve occasional clumps of nuclei in the thicker parts of the syncytium. The nuclear basophilia is attributable to desoxyribonucleoproteins. After the basophilic substance has disappeared, the nuclei can still be recognized for a period by an affinity of their underlying structural proteins for acid dyes, but eventually many of these nuclei disappear completely. Pari passu with the diminution of the nuclear basophilia, shortly before term, acid phosphatase appears in the nuclei in appreciable quantities. Thus, briefly stated, one of the major histochemical characteristics of the ageing placenta is the gradual diminution in cytoplasmic nucleoprotein and the gradual rise in both acid and alkaline phosphatases.

The Histochemistry of the Placental Villi from two Cases diagnosed as Severe Preeclampsia and as Eclampsia. The placentas on which these observations were carried out were from two cases in which diagnoses were reached of severe preeclampsia and eclampsia. The first patient had no convulsions but showed marked hypertension, albuminuria and edema of the extremities, the only unusual feature being that the condition was present at four and a half months of gestation. Pregnancy was terminated artificially because of the threat to the mother's life. The second patient, in addition to hypertension,

albuminuria and edema, had a succession of convulsions which did not subside until the fetus had been eliminated. Interestingly, this case also occurred between four and five months after the onset of pregnancy.

Although both of these placentas were obtained at four and one-half months of gestation, they had many characteristics of much older placentas. Many of the terminal villi showed a thinning of the trophoblast covering them so that appreciable areas of the syncytium were reduced to thin structureless membranes exhibiting a distinct affinity for acid dyes (figs. 8 and 12). In some areas the trophoblast so transformed resembled the normal syncytial plates of the placenta at term. Nevertheless, in many of the villi, the process involved much larger areas of syncytium than is the case in the normal placenta at term. Cytoplasmic basophilia had entirely disappeared from these hyaline areas and the few nuclei which were present also showed a diminished affinity for basic dyes. Some of the very tips of the terminal villi gave the appearance of having undergone complete hyaline degeneration including the stroma and capillaries within them; such villi were visible as small acidophilic objects which possessed little discernible structure. This feature was more apparent in the first than in the second specimen.

Besides the villi described above with predominantly hyaline syncytium and diminished basophilia, many other terminal villi, as well as the majority of the larger villi, showed scarcely any noticeable change. Their nuclei stained intensely blue and the syncytial cytoplasm contained amounts of basophilic substance corresponding to normal placentas of four and a half months. Intensely blue-staining sproutlike tags of syncytium occurred in normal numbers.

The most striking histochemical feature of both of these specimens was the abnormally great quantity of both alkaline and acid phosphatases present within the syncytium clothing the villi (figs. 7,

EXPLANATION OF PLATE 3

FIG. 10. Chorionic villi from the same case of preeclampsia at 4½ months illustrating the abnormal density of alkaline phosphatase. Note, in one place where the syncytium has become completely hyaline (lower center), that phosphatase is absent. ×280.

FIG. 11. Chorionic villi from the same case of preeclampsia, illustrating the extremely premature accumulation of acid phosphatase in the syncytium. Observe that there is little or no phosphatase in the thinned out hyaline areas of which there are abnormally many for a placenta of 4½ months. ×250.

FIG. 12. Chorionic villi from the same preeclamptic case. Zenker acetic fixation. Eosin-methylene blue stain. Observe the villi with an abnormal degree of hyaline transformation of the trophoblast for a placenta of 4½ months. These thinned out, hyaline places are more extensive than normal, contain few nuclei, and have an affinity for acid dyes. ×350.

FIG. 13. Chorionic villi from the same preeclamptic case, stained for glycogen by Bauer's stain, illustrating the abnormal increase in glycogen (black) within the stroma of the villi. The abnormal distribution of glycogen here differs materially from its abnormal occurrence in hydatidiform villi; in the latter, glycogen is not very abundant in the degenerated stroma of the villi, but is, on the contrary, markedly increased in the trophoblast. ×250.

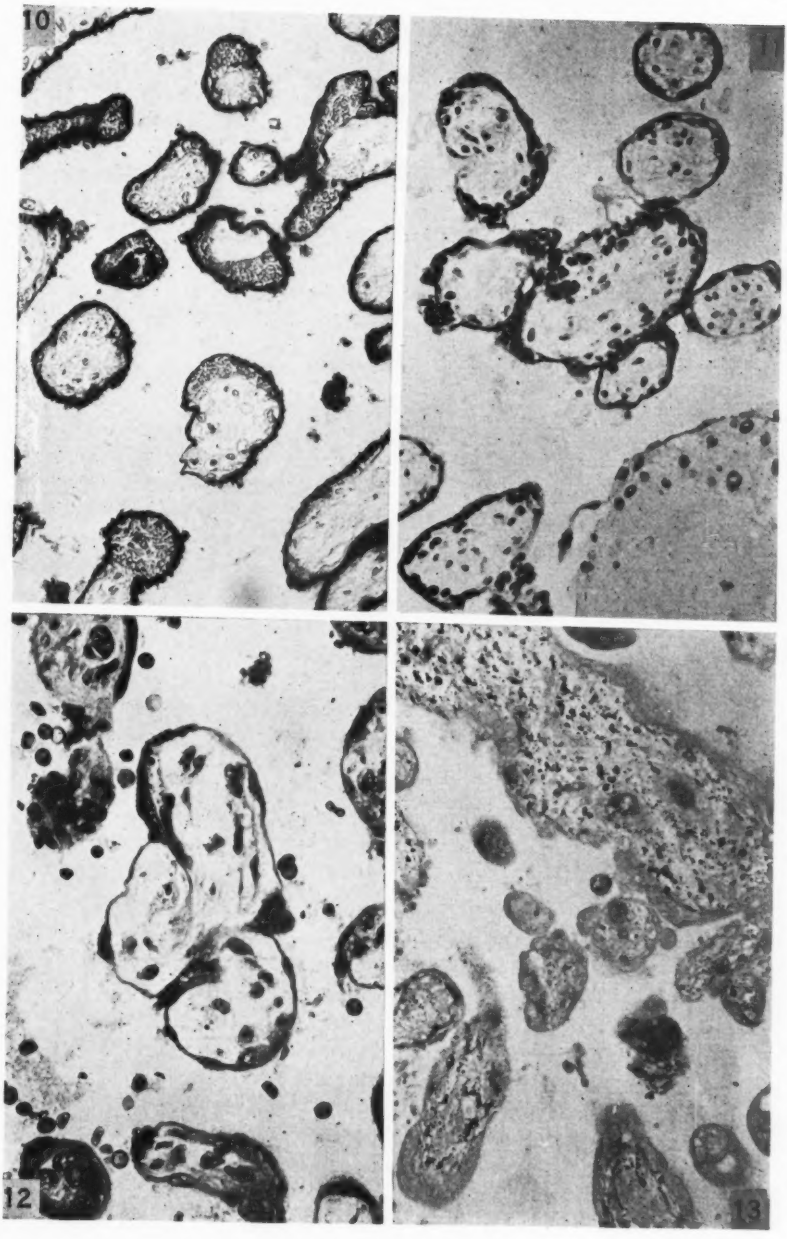

PLATE 3

9, 10 and 11). In respect to alkaline phosphatase the picture was one of a placenta from the last third of gestation rather than at four and a half months (cf. figs. 5 and 7). Alkaline phosphatase was present in conspicuously abnormal amounts in the cytoplasm of the syncytial trophoblast of the great majority of villi (fig. 10). Acid phosphatase was present in maximal concentration in the nuclei of the syncytium (figs. 7 and 11) as well as to some extent in the perinuclear cytoplasm. It should be recalled that in the normal placenta acid phosphatase does not appear until shortly before term. Thus both of these placentas contained abnormal quantities of phosphatase, although the second, derived from the case of eclampsia, contained more than the first; we mention this without wishing to attach any special significance to it.

The Bodian protargol reaction revealed the presence of argyrophil material in both the syncytium and stroma of many of the villi. In normal placentas of four and five months argyrophil material is confined exclusively in the stroma in the form of a subepithelial layer (vide ante). In our two eclamptic specimens, on the contrary, argyrophil particles were scattered throughout both the syncytium and stroma of many villi, thus differing from the normal placenta.

Sections of the specimens stained for glycogen revealed a moderate increase of this substance in the stroma of a number of the medium sized and larger villi (fig. 13). No glycogen was present, however, in the trophoblast covering these same villi.

The placentas contained numerous cell islands and remains of the trophoblastic shell and cell columns. The cytotrophoblast comprising these structures contained ample complements of basophilic substance and glycogen and did not appear to be noticeably abnormal. The Langhans cells of the chorionic villi, on the contrary, seemed to us to be too few in number for placentas of four and a half months, the cells having regressed prematurely along with the associated changes in the syncytium.

In the half dozen blocks taken at random from the first placenta we were impressed by the number of areas in which small infarctions had occurred. In such areas the maternal blood in the intervillous space appeared to have stagnated and to have clotted. In these areas the syncytium covering the villi seemed to be rather generally necrotic and contained excessive amounts of alkaline phosphatase (fig. 9). These particular features were not evident in the second placenta. Phosphatase was also prominently in evidence in the stagnated blood in the intervillous space in such areas. In the preeclamptic placenta, also, some of the villi appeared to be somewhat edematous, a feature which was not observed in the second specimen.

Comments on the Placenta in Eclampsia. In placentas of two cases diagnosed respectively as severe preeclampsia and eclampsia we have

observed that in the trophoblastic syncytium, acid and alkaline phosphatases are prematurely increased while basophilia has appreciably diminished.

Phosphatases very possibly play a rôle normally in the transfer of carbohydrates and lipoids across the placental barrier. Moreover, they probably remove phosphate from nucleoproteins (Dempsey and Wislocki, '45), being responsible possibly for the gradual decline of ribonucleoprotein in the syncytium. The fact that basophilia decreases prematurely in eclamptic placentas, whereas phosphatases accumulate at an accelerated rate, suggests a possible disturbance of nucleoprotein metabolism in this disease.

We do not know whether the phosphatase which accumulates normally in the syncytium is actually formed there or is merely brought to the placenta by the maternal circulation to be filtered out. It is known merely that alkaline phosphatase increases steadily in amount in the maternal blood plasma during gestation (Bodansky, Campbell and Bell, '39) and that it first appears in the brush-border of the syncytium and subsequently advances inward.

In eclamptic villi we have observed widely disseminated argyrophil material in the syncytium and stroma as well as a perceptible increase of glycogen in the stroma. In villi of hydatidiform moles, on the contrary, we have found little, if any, argyrophil material but a tremendous storage of glycogen in the trophoblast covering the villi. It should be recalled that we have adduced evidence earlier in this paper, as well as in previous ones (Wislocki and Dempsey, '45), for believing that the argyrophil material displayed in chorionic villi by the Bodian protargol method should be regarded as formed calcium. Moreover, in a previous section of this paper we offered reasons indicating that the absence of calcium in the villi of hydatidiform moles and the marked increase in glycogen should be attributed to the absence of a fetal circulation in the villi in addition to a probable impairment of the maternal circulation bathing the villi. The atypical distribution and abnormal amounts of calcium and glycogen in the villi of the eclamptic placenta indicate, we believe, that there is probably some impairment of the blood supply of the chorionic villi in this disease also. The abnormal accumulation of glycogen indicates, in our estimation, the presence of a certain degree of anoxemia (Cf. Dempsey and Wislocki, '44).

In closing we wish to mention a placenta at term of a patient suffering from renal hypertension and nephritis. This placenta, unlike the eclamptic ones, showed no abnormality in reference to cytoplasmic basophilia, phosphatase or ordinary histology. Consequently, the premature ageing and abnormal accumulation of phosphatases which we have described in eclamptic placentas distinguish them from a placenta from a case of nephritis.

SUMMARY

Acid phosphatase accumulates in the trophoblastic syncytium of the human placenta towards the very end of gestation; it occurs principally in the nuclei of the syncytium and to a lesser extent in the perinuclear cytoplasm. Alkaline phosphatase begins to appear in the syncytium in the first months of pregnancy and increases steadily until term. Thus these two enzymes characterize the ageing placenta; they occur in inverse proportion to cytoplasmic basophilic substance (ribonucleoprotein) which diminishes steadily as the placenta ages.

Iron and calcium are demonstrable in considerable quantities in the first half of gestation in the stroma of the chorionic villi, where they are mainly deposited just beneath the trophoblastic epithelium. In the same localities and during the same period traces of acid and alkaline phosphatases are also encountered.

The histochemical reactions of two hydatidiform moles have been investigated. The viable syncytium of the moles contains numerous birefringent fat droplets, exhibits a greenish fluorescence when examined in ultraviolet light and gives a positive reaction with Schiff's plasmal reagents. The combination of these reactions indicates that the syncytium is producing placental steroid hormones, a conclusion consonant with the known excretion of steroid hormones in the presence of hydatidiform moles. No calcium and only diffuse traces of iron were demonstrable in the molar villi by the Bodian protargol reaction and the Turnbull blue method. No acid phosphatase and relatively little alkaline phosphatase were found in the moles, but glycogen was present in both the cytotrophoblast and syncytium in amounts greatly increased over normal. As explained in the text, the increases or decreases of these various substances, in contrast to normal villi, are referable to the absence of the fetal circulation and a diminished maternal circulation.

Two placentas from cases diagnosed respectively as severe pre-eclampsia and as eclampsia were subjected to cytological and histochemical examination. Both cases were atypical in so far as in each the condition was present at four and a half months of gestation. The chorionic villi were prematurely aged as judged by their cytological and histochemical appearance. Many of the villi showed premature ageing of the syncytium (thinning, loss of basophilia, increased affinity for acid dyes, and some deterioration of nuclei), while a few of the very smallest terminal villi showed complete necrosis of both stroma and syncytium. Ribonucleoproteins (cytoplasmic basophilia) were prematurely reduced, whereas alkaline and acid phosphatases were appreciably increased. There were also noticeable changes in the amount and distribution of both calcium and glycogen. The decrease in cytoplasmic basophilia associated with a premature increase in phosphatases suggests a possible disturbance of nucleoprotein metabolism in this disease.

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THE RELATION OF THE HYPOPHYSIS TO CERTAIN CHANGES INDUCED IN THE RAT BY THE GOITROGEN, PROMIZOLE[1]

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A NEW IMPETUS was given to the study of the cytology and physiology of the thyroid when it was observed that certain compounds, when administered to animals, produce marked enlargement of their thyroid glands. Kennedy and Purves (1941) had shown that when animals are fed a Brassica seed diet, extreme hyperplasia of the thyroid glands ensues. Astwood, Sullivan, Bissell, and Tyslowitz (1943) and MacKenzie and MacKenzie (1943) showed that certain sulfonamides and thiourea-like drugs not only induce enlargement of the thyroid but lower the basal metabolic rates of animals as well. Later Griesbach and Purves (1943), Astwood and Bissell (1944), Franklin, Lerner and Chaikoff (1944), and Rawson, Tannheimer and Peacock (1944) showed clearly that these drugs interfere with the metabolism of iodine and that the thyroids of animals receiving them are unable to take up iodine or synthesize thyroxine. Supplemental iodine, even in large amounts, proved ineffective in preventing the changes in the thyroid induced by these drugs or by the Brassica seed diet, while thyroxine in relatively small daily amounts completely inhibited them.

It was recently shown (Higgins and Larson, 1944; Higgins, 1945) that the sulfone, promizole, hitherto shown (Feldman, Hinshaw and Mann, 1944) to exert a favorable influence on the extent of experimental tuberculosis of guinea pigs, is likewise goitrogenic. The administration of promizole, either parenteral or oral, to rats produced thyroid changes which resembled those which ensued when other goitrogens were given. These changes included the vacuolation and destruction of the colloid bodies, marked increases of the height of thyroid cells and increases of the number of thyroid acini, all resulting in greatly increased weight of the thyroid gland. These changes were

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[1] Promizole (4,2′-diaminophenyl-5′-thiazolyl sulfone) was provided through the courtesy of Dr. E. A. Sharp and Dr. L. A. Sweet of Parke, Davis and Co., Detroit, Michigan.

likewise completely inhibited by thyroxine but iodine, given in even larger amounts, did not prevent them.

MacKenzie and MacKenzie (1943), as well as Astwood, Sullivan, Bissell and Tyslowitz (1943), stated that the changes which occur in the thyroids of intact animals when sulfaguanidine or thiouracil is given do not develop in animals which have been previously hypophysectomized. This would indicate that the effects of the drug on the thyroid are mediated through the pituitary gland. An increased elaboration of thyrotropic hormone by the anterior lobe may occur in response to the decreased output of thyroxine by the thyroid. Cytologic changes in the basophilic cells of the anterior lobe, such as occur on thyroidectomy, were observed by Griesbach and Purves (1943) and by MacKenzie and MacKenzie (1943). Subsequently, however, Williams, Weinglass, Bissell and Peters (1944) could not identify any histologic changes in the pituitary glands of animals receiving thiouracil.

Results of bio-assay studies of the thyrotropic hormone content of the pituitary glands and serums of goitrous animals have not been entirely consistent. Gordon, Goldsmith and Charipper (1945) concluded that the amounts of thyrotropic hormone in the glands and the serums of goitrous rats are less than in control animals. But Griesbach and Purves (1943) concluded that the serums of goitrous rats are significantly enriched in thyrotropic activity when compared with serums of controls. However, they reported that the thyrotropic contents of the pituitaries of their goitrous rats was lower than that of control animals, although an increase of the percentage of basophilic cells in the anterior lobe had occurred.

Although the evidence implicates the pituitary in inducing the thyroid changes, a complete report of the effects of these newer goitrogens on the thyroid gland after removal of the pituitary has not appeared. The present report covers the results of a study of the administration of promizole (4,2′-diaminophenyl-5′-thiazolyl sulfone) to normal adult male white rats and to a series of adult male white rats of the same age and strain which had been previously hypophysectomized. Since promizole has been shown to exert certain toxic effects on the blood, this report includes data assembled from studies on the formed elements of the blood in normal and hypophysectomized animals given this goitrogen.

METHOD

Thirty-two adult male white rats of the Sprague-Dawley strain weighing from 200 to 225 gm. were selected. They were fed our standard laboratory ration. Sixteen of them were hypophysectomized and sixteen were left intact. All hypophysectomized animals were given daily 0.2 to 0.5 cc. of phyone, an anterior pituitary[2] extract. Three weeks after hypophysectomy, twelve

[2] Prepared, assayed and provided through the courtesy of Dr. David Klein, Wilson Laboratories, Chicago, Illinois.

hypophysectomized and twelve intact animals were given, intraperitoneally, 15 mg. of promizole as a suspension in distilled water each day for twenty-one days. Four hypophysectomized and four intact animals did not receive the drug but served as controls. The drug was given intraperitoneally to insure an equal amount of the goitrogen to each animal. When the drug is included in the diet, its intake will depend on appetite, which is often variable. We had previously learned that promizole is as effective a goitrogen when given parenterally as by mouth.

Using a closed circuit method previously described (Chapman, Baldes and Higgins, 1944), the oxygen consumption of all animals was recorded in calories per square meter per hour before and after the goitrogen was given.

Samples of blood were taken from the heart of each test and control animal, before and after the twenty-one day experimental period. The numbers of erythrocytes per cubic millimeter of blood, their volumes in cubic microns, the grams of hemoglobin per 100 cc. of blood, the number of leukocytes per cubic millimeter and their differential distribution were determined and statistically computed. At the end of the experiment all control and test animals were killed and their thyroid glands were removed, weighed on a precision balance, fixed in formalin and prepared for histologic study.

RESULTS

The data assembled on the average weights of all animals, on the average weights of their thyroid glands and on the average weights

TABLE 1. BODY WEIGHTS, AND WEIGHTS OF THYROID GLAND AND PITUITARY GLAND AFTER TWENTY-ONE DAILY INJECTIONS OF PROMIZOLE INTO TEST ANIMALS

Group		Animals	Average body weight, gm.	Promizole, mg. daily*	Thyroid gland, mg.		Pituitary gland, mg.	
					Absolute	Per 100 gm. body wt.	Absolute	Per 100 gm. body wt.
Intact	Control	4	289.5	0	11.2±0.2†	3.9±0.2	7.0±0.2	2.5±0.1
	Test	12	293.4	15	25.7±1.3	8.9±0.4	8.0±0.1	2.7±0.1
Hypophysectomized	Control	4	180.0	0	11.2±0.2	6.3±0.2		
	Test	12	171.9	15	9.9±0.7	5.8±0.4		

* Intraperitoneally.
† Probable error of the mean.

of the pituitary glands of the intact animals are condensed into table 1. The intact control and test animals gained weight during the three week period, while the hypophysectomized control and test animals all lost weight. Hypophysectomized animals given the drug lost slightly more weight than those which did not receive it.

Hyperplastic thyroid glands developed in all intact animals given the drug. The mean weight of the thyroid gland of these animals

(25.7±1.3 mg.) was not large and yet it was more than twice the average weight of the thyroid gland of their controls (11.2±0.2 mg.). The colloid bodies of the acini of the thyroids of intact animals receiving the goitrogen had practically disappeared and the heights of the acinar cells (fig. 1) were greatly in excess of those of the acini of normal thyroids. The mean weight of the thyroid glands (9.9±0.7

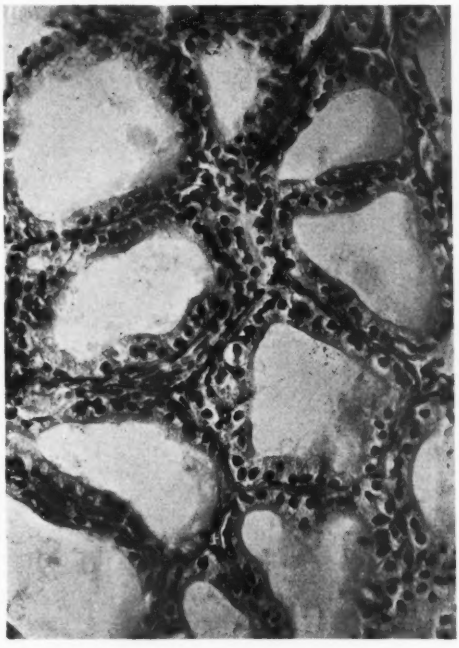

FIG. 1. Section of thyroid acini of normal animal which had received the goitrogen daily for twenty-one days (hematoxylin and eosin ×210).

mg.) of the hypophysectomized test animals was statistically like that (11.2±0.2 mg.) of hypophysectomized animals which had not received the drug. Thus the goitrogen, promizole, when given to the hypophysectomized rats, did not increase the weight of the thyroid gland nor induce thyroid hyperplasia (figs. 2 and 3). Thyroid cells were flat and the cellular architecture of the gland resembled that of the thyroid of an hypophysectomized animal which had not received the drug (fig. 4). Although the goitrogen did not change the epithelial patterns of the thyroid gland when given to hypophysectomized animals, yet the effect on the colloid bodies was as marked as in intact control animals. Normal colloid patterns were observed in the peripheral acini of the gland (fig. 2) but throughout the center, the acini

were completely devoid of colloid or contained residual shreds of it. Obviously the effect of the goitrogen on the stored thyroid reserves in hypophysectomized animals was quite like that seen when intact animals were given the drug.

Three weeks after the pituitary gland had been removed, the average oxygen consumption of all hypophysectomized animals, computed in calories per square meter per hour, was 28.1 per cent less than the average of all of the intact control animals. This depression of the metabolic rate was presumably due at least in part to the loss of the

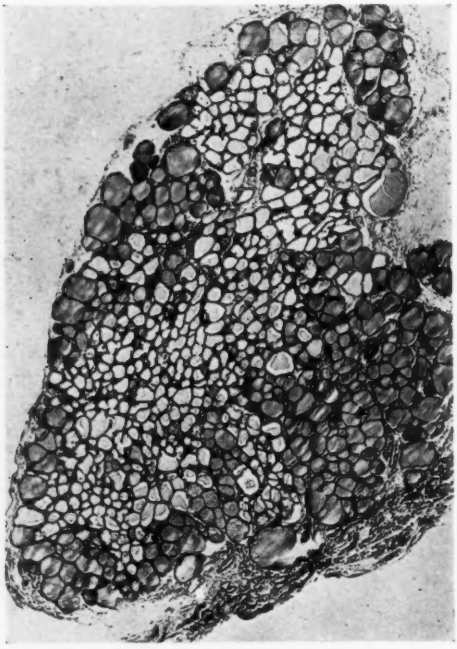

FIG. 2. Section of thyroid gland of animal hypophysectomized and given the goitrogen, promizole (hematoxylin and eosin ×40).

influence of the pituitary factor on thyroid function but other systemic effects of hypophysectomy undoubtedly were additional contributing factors. Thyroid activity, although suppressed, was not entirely inhibited by the loss of the pituitary.

Three weeks later, after 15 mg. of promizole had been given intraperitoneally each day to the two test groups of twelve animals each, the average oxygen consumption per rat of the intact control group of four animals was 53.8 ± 1.1 calories per square meter per hour. The average oxygen consumption per rat of the intact group of twelve animals which had received the goitrogen and in which goiters had

developed was 44.6 ± 0.9 calories per square meter per hour. This figure was 9.2 ± 1.4 calories per square meter per hour less and represents a decline of the metabolic rate of 17.1 per cent.

At the same time the average oxygen consumption of the untreated hypophysectomized animals was 38.6 ± 1.2 calories per square meter per hour, which was 28.3 per cent less than the average oxygen consumption of the untreated control animals. The average consumption by hypophysectomized rats which had received promizole was 33.8

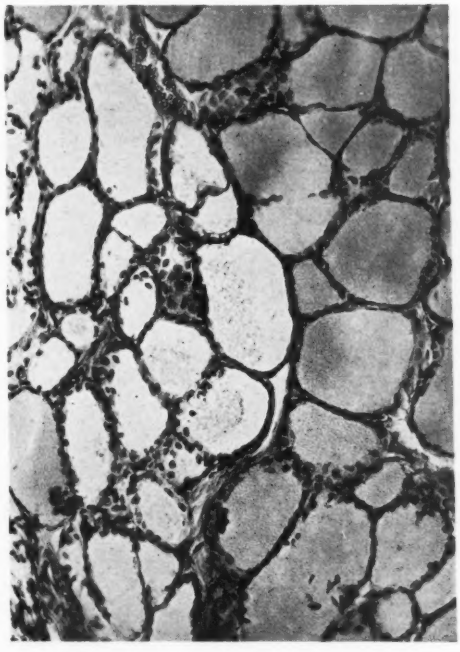

FIG. 3. Section of thyroid acini of the same gland shown in figure 2 (hematoxylin and eosin ×210).

± 2.2 calories per square meter per hour. Thus the oxygen consumption by hypophysectomized animals receiving the goitrogen was only 4.8 ± 2.5 calories per square meter per hour (12.4 per cent) less than that of their controls. It appears therefore, that the drug had not exerted a further suppression of thyroid function beyond that exerted by the removal of the pituitary.

The hematologic data assembled from samples of heart blood of the intact and hypophysectomized control and test animals at the end of the experiment are condensed into tables 2 and 3. Three weeks after removal of the hypophysis and before promizole was given to the test groups, the average total erythrocyte count was 1,400,000

$\pm$400,000 cells per cubic millimeter of blood less than that of their intact controls. The concentration of hemoglobin was correspondingly reduced. But significant differences of either erythrocyte volumes or the total number of leukocytes per cubic millimeter of blood were not apparent between the hypophysectomized and the intact groups. The percentage of granulocytes in the blood of the hypophysectomized group was less, however, than that recorded for intact animals.

Three weeks later, after promizole had been given to the two test groups (table 2) the average erythrocyte count in the hypophysectomized control animals was 1,300,000$\pm$600,000 cells less than in

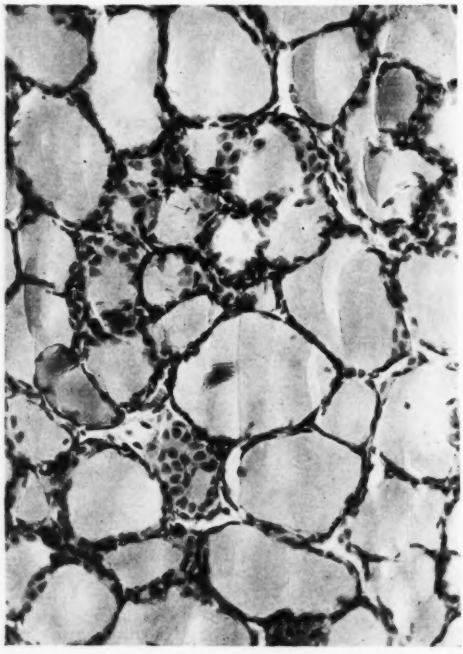

FIG. 4. Section of thyroid acini of animal hypophysectomized three weeks before (hematoxylin and eosin $\times$210).

their intact controls, a difference probably not significant. The average volume, in cubic microns, of the erythrocytes, as well as the average total number of leukocytes per cubic millimeter of blood, did not differ significantly in the two control groups of animals. The average hemoglobin value in the hypophysectomized control animals, however, was significantly less than the average obtained in the intact group.

In both intact and hypophysectomized animals promizole induced marked destruction of erythrocytes. In the intact group, those re-

ceiving the drug had an average erythrocyte count 2,800,000 ± 200,000 cells per cubic millimeter of blood less than their controls, while in the hypophysectomized group, those receiving the drug had an average erythrocyte count 2,300,000 ± 600,000 cells less than their controls. There were significant reductions of the grams of hemoglobin per 100 cc. of blood, from their controls, in both groups of test animals which had received the drug during the three week period but a significant increase in the average size of the erythrocytes was observed in the intact group only. The total leukocyte count was not affected when the goitrogen was given to the intact animals but it was significantly elevated by the drug in the hypophysectomized animals.

TABLE 2. SUMMARY OF HEMATOLOGIC DATA

Group		Animals	Erythrocytes, millions per cu. mm.	Erythrocytes, volume in cu. microns	Hemoglobin, gm. per 100 cc. of blood	Leukocytes, thousands per cu. mm.
Intact	Control	4	10.1 ± 0.1*	47.3 ± 0.2	17.1 ± 0.1	12.1 ± 0.8
	Test†	12	7.3 ± 0.2	53.9 ± 1.7	13.8 ± 0.2	11.1 ± 0.9
Hypophysectomized	Control	4	8.8 ± 0.6	41.8 ± 3.6	13.6 ± 0.2	10.2 ± 0.3
	Test†	12	6.5 ± 0.1	49.5 ± 1.1	11.1 ± 0.4	18.3 ± 1.8

* Probable error of the mean.
† Received 15 mg. of promizole daily intraperitoneally for twenty-one days.

TABLE 3. DIFFERENTIAL DISTRIBUTION OF LEUKOCYTES (PER CENT)

Group		Animals	Lymphocytes	Monocytes	Granulocytes		
					Neutrophilic	Eosinophilic	Basophilic
Intact	Control	4	75.0 ± 1.4*	1.1	21.0 ± 1.3	2.2	0.7
	Test†	12	78.6 ± 2.2	1.5	17.4 ± 1.6	2.1	0.4
Hypophysectomized	Control	4	81.5 ± 0.2	1.5	14.5 ± 0.7	2.5	0
	Test†	12	73.1 ± 4.2	0.5	25.2 ± 4.4	0.8	0.4

* Probable error of the mean.
† Received 15 mg. of promizole daily intraperitoneally for twenty-one days.

Significant differences between the lymphocyte percentages of either the intact or the hypophysectomized animals as a result of giving the goitrogen were not observed (table 3). Likewise the percentages of monocytes, eosinophilic and basophilic granulocytes were unchanged as a result of administration of the goitrogen but there was a considerable increase of the percentage of neutrophilic granulocytes in the hypophysectomized animals which received the drug. However, the variability of the data is such as to indicate that the administration of promizole, in the amounts given, did not alter the

differential distribution of the leukocytes in either normal or hypophysectomized animals.

COMMENT

Promizole (4,2′-diaminophenyl-5′-thiazolyl sulfone), when given to experimental animals, either parenterally or by mouth, will incite changes in the thyroid similar in character to those induced by thiourea, thiouracil or certain sulfonamides. The extent of these changes in the thyroid, such as destruction of colloid and cellular hyperplasia, depends on the amounts of the drug given and the duration of the period of administration. Such changes in the thyroid were not prevented by giving large amounts of iodine but were entirely inhibited by giving even small amounts of thyroxine during the period when promizole was provided.

Although we do not have direct evidence, the action of promizole is considered to be like that of other goitrogens wherein the metabolism of iodine and the synthesis of thyroxine are interrupted by the failure of the thyroid to collect iodine and to convert it into diiodotyrosine. It has been shown elsewhere that much smaller percentages of radioactive iodine were taken up by thiouracil-stimulated thyroids than by normal thyroid glands or by those stimulated by potassium thiocyanate. Although our own data on the collection of radioactive iodine by promizole-stimulated thyroids are still incomplete we have reason to believe from histologic evidence at least that the action of promizole is like that of thiouracil. The decrease in the rate of oxygen consumption by promizole-treated rats was evidence too that the synthesis of thyroxine had been at least partially interrupted. In an earlier study (Higgins, 1945) it was observed that the average rate of oxygen consumption of rats given 15 mg. of promizole daily for six weeks was 25.4 per cent lower than that of their controls. Thyroid activity was only partially suppressed by this amount of promizole; for in spite of considerable degrees of thyroid hyperplasia we have encountered rates of oxygen consumption which were well within the normal range.

The hyperplasia of the thyroid, following the administration of either sulfaguanidine or thiouracil, as shown elsewhere was clearly of pituitary origin; for goiters did not develop after hypophysectomy. This must indicate that the hyperplasia was the result of an increased elaboration of the thyrotropic principle of the anterior lobe. We have observed real increases of the weights of the pituitary glands of immature animals made goitrous by promizole but we have not encountered consistent increases of pituitary weights in adults. In histologic sections we have seen increased numbers of vacuolated basophilic cells; as did MacKenzie and MacKenzie (1943) and Griesbach and Purves (1943). Williams, Weinglass, Bissell and Peters (1944), however, did not detect histologic changes in the pituitary glands of animals which had received thiouracil.

Promizole, when given to animals previously hypophysectomized, appeared to exert an effect on the colloid bodies of their thyroids which resembled that observed when the drug was given to intact animals. This effect appeared to be a direct one, wherein the drug acts in such manner as to induce destruction of the thyroid reserves. However, the secondary or indirect effect of the drug namely, the thyroid-stimulating effect, did not occur in the absence of the pituitary. The thyroid acinar cells remained flat and resembled those of glands of hypophysectomized animals which were not given the goitrogen. Whereas there were marked statistical differences between the weights (both absolute and relative) of the thyroid glands of intact animals receiving the goitrogen and their untreated controls, no such differences were encountered in animals from which the pituitaries had been removed.

The removal of the pituitary exerted a considerable effect on the rate of oxygen consumption. During the three week period between the time of operation and the time administration of the goitrogen was begun, the average oxygen consumption by the hypophysectomized animals was 28.1 per cent less than the average recorded for the intact group. Three weeks later after the drug had been given to the test animals there was no further decline in rate; for the average oxygen consumption by the four hypophysectomized animals was then 28.3 per cent less than the average of the four intact animals. The goitrogen, when given for three weeks to the twelve intact animals, reduced the average consumption to a level 17.1 per cent below that of their controls; this difference was of statistical significance. But when the drug was given to the twelve hypophysectomized animals, the average oxygen consumption, although reduced by 12.4 per cent from their control level, was not significantly different from the average recorded for the four hypophysectomized animals which had not received the drug. Thus it would appear that in hypophysectomized animals the drug did not further suppress thyroid function from levels which hypophysectomy alone had induced.

We gave phyone to all hypophysectomized animals as substitution therapy before and during the time they were given promizole. Astwood, Sullivan, Bissell and Tyslowitz (1943) gave 25 mg. of powdered whole pituitary of sheep to their hypophysectomized animals. Phyone was first prepared by Van Dyke and Wallen-Lawrence (1930), who demonstrated its growth-promoting potencies. Shockaert (1931) induced in young ducks a thyroid response with phyone and observed lowered thyroid iodine levels. A gonadotropic action of phyone was demonstrated by Hertz, Hellbaum and Hisaw (1932). Furthermore phyone elevated glycogen stores in liver and muscle and prevented hypoglycemia (Soskin, Levine and Lehmann, 1939). Phyone in the amounts given to our hypophysectomized animals, did not cause any stimulation of the thyroid epithelium (fig. 4), although Astwood and

his co-workers (1943) did observe slight enlargements of the thyroids of their hypophysectomized animals which had received the powdered pituitary as substitution therapy. Unfortunately, we did not maintain hypophysectomized animals without phyone; so that we can not appraise the effects of phyone, per se, on the cytologic structure of the thyroid or on the rates of oxygen consumption.

The toxic changes which promizole had been previously shown to exert on the blood of experimental animals were equally manifest in these animals from which the pituitaries had been removed. Hypophysectomy in itself induced mild anemia, a condition previously described by Meyer, Thewlis and Rusch (1940) and by Vollmer, Gordon and Charipper (1942). The further destruction of blood, caused by promizole, was quite like that which occurred when intact animals were given the drug. In the intact animals the erythrocyte count was $2{,}800{,}000 \pm 200{,}000$ cells per cubic millimeter of blood less than that of their controls, while in hypophysectomized animals given the drug the erythrocyte count was $2{,}300{,}000 \pm 600{,}000$ cells per cubic millimeter less than that of their controls. Corresponding decreases of the concentration of hemoglobin were 3.3 ± 0.2 and 2.5 ± 0.4 gm. per 100 cc. of blood, respectively.

SUMMARY

A study of the action of the goitrogen, promizole, on the thyroid glands, the rate of oxygen consumption and the blood, of hypophysectomized rats is reported. The toxic effects of the drug on the colloid bodies of thyroid acini were as manifest in hypophysectomized animals as in intact animals but hyperplasia of the thyroid cells and acini did not occur when the pituitary had been removed. Hypophysectomy in itself lowered the rate of oxygen consumption but the administration of the goitrogen to such hypophysectomized animals did not further reduce thyroid activity as judged by the rate of oxygen consumption. Hypophysectomy induced mild anemia and the administration of promizole to such hypophysectomized animals further reduced the erythrocyte and hemoglobin levels in amounts essentially equivalent to the reductions in these categories observed in normal animals when given the same amounts of the drug.

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THE INFLUENCE OF HYPOPHYSECTOMY UPON PLASMA IODINE AND THYROXINE CONTENT OF THE THYROID GLAND OF THE RAT[1]

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IN AN EARLIER STUDY (Morton, Perlman, Anderson, and Chaikoff, 1942) radioactive iodine was employed to investigate the turnover of iodine by the thyroid gland of the hypophysectomized rat. The results brought to light an interesting difference in the fate of radioactive inorganic iodine injected into normal and hypophysectomized rats. The normal thyroid gland rapidly converted labeled inorganic iodide to diiodotyrosine, and this conversion was also found to occur readily in the thyroid gland of the rat subjected to hypophysectomy. But the rate of formation of radio-thyroxine by the thyroid gland was greatly depressed by hypophysectomy.[2]

For a more complete understanding of the effects of hypophysectomy upon iodine metabolism, it became necessary to provide reliable data on the thyroxine content of the thyroid gland and on the protein-bound iodine of plasma of the hypophysectomized rat. This has been done in the present investigation.

EXPERIMENTAL

Male rats weighing 225–300 gms. were hypophysectomized by the parapharyngeal route. After this operation they were fed a stock diet that ensured an average intake of about 3 gamma of iodine per day. It had the following composition: wheat, 68.5 per cent; casein, 5 per cent; fish meal, 10 per cent; alfalfa, 10 per cent; sodium chloride, 1.5 per cent; fish oil, 5 per cent.

Blood was removed by heart puncture, the rats being anesthetized with nembutal during this procedure. All animals were exsanguinated before excision of their thyroid glands. In order to provide enough material for analyses of the various iodine fractions, the plasma of two rats was combined, as were also the thyroid glands of the same two rats.

Total and protein-bound iodine of plasma were determined on 3-cc.

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[2] In referring to this investigation, Baumann, Metzger, and Marine (1943) have stated that "the thyroxine content of thyroids of hypophysectomized rats was reduced to a minimum." Since no chemical (i.e. I^{127}) analyses were presented, this statement is not a valid interpretation of the radioactive measurements made by Morton, Perlman, Anderson, and Chaikoff (1942).

samples of plasma by a modification of the Chaney method (Taurog and Chaikoff). The procedure used for the fractionation of thyroid iodine has also been described elsewhere (Taurog and Chaikoff).

Each value recorded in Table 1 represents an analysis of the pooled thyroid glands or plasma from two rats. The hypophysectomized rats were

TABLE 1. EFFECT OF HYPOPHYSECTOMY ON THYROID AND PLASMA IODINE

Experiment	Days after Hypophysectomy	Weight of rats		Weight of thyroid glands[1]	Thyroid weight per 100 gm. body weight	Thyroid gland					Plasma	
						Thyroxine iodine		Total iodine		Thyroxine iodine as per cent of total iodine		
		At start[1]	At end[1]			Present in glands[1]	Per cent of wet gland	Present in glands[1]	Per cent of wet gland		Protein-bound iodine[1]	Total iodine[1]
		gm.	gm.	mg.		micrograms	per cent $\times 10^3$	micrograms	per cent $\times 10^3$		micrograms per 100 cc. plasma	micrograms per 100 cc. plasma
1 Control	1		288								3.3	
Operated	1		290								3.1	
2 Control	2		282	29	10.3						3.1	
Operated	2		266	23	8.6						2.2	
3 Control	2		269	35	13.0	2.3	6.6	6.8	19	34	2.7	
Operated	2		260	26	10.0	2.2	8.5	6.8	26	32	2.3	
4 Control	3	262	250	31.5	12.6	2.6	8.3	9.3	30	28	2.8	2.6
Operated	3	260	234	22.5	9.6	2.6	12	9.9	44	26	1.6	1.3
5 Control	3	258	240	22	9.2	1.9	8.6	6.9	31	28	2.6	3.2
Operated	3	261	235	22	9.3	2.4	11	9.2	42	26	1.3	1.5
6 Control	4	255	243	34.5	14.2	2.8	8.1	9.6	28	29	2.5	2.5
Operated	4	263	238	27.5	11.5	3.7	13	12.3	45	30	1.3	1.4
7 Control	4	242	232	30	12.9	2.5	8.3	7.5	25	33	2.6	2.4
Operated	4	270	240	26	10.8	3.4	13	11.5	44	30	1.1	1.0
8 Control	9	259	246	25	10.2	2.1	8.4	7.9	32	27	3.2	3.1
Operated	9	256	214	17	7.9	2.2	13	9.2	54	24	0.9	1.5
9 Control	9	263	236	27.5	11.6	1.7	6.2	5.9	21	29	2.0	2.2
Operated	9	261	235	16.5	7.0	2.3	14	8.0	49	29	1.2	1.5
10 Control	24	227	227	21.5	9.5	2.2	10	8.5	40	26	3.0	
Operated	24	243	181	12.5	6.9	2.6	21	10.5	84	25	1.2	
11 Control	24	237	205	18.5	9.0	2.1	11	8.3	45	25	2.9	3.1
Operated	24	242	201	16	8.0	2.8	17	12.4	78	23	1.6	1.7
12 Control	44	235	227	23.5	10.3	2.6	11	8.8	37	30	3.6	
Operated	44	238	201	13.5	6.7	3.1	23	9.0	67	34	1.9	
13 Control	44	246	242	24	9.9	2.9	12	9.3	39	31	3.4	
Operated	44	251	200	13.5	6.7	3.1	23	11.4	84	27	1.9	
14 Operated	1 yr.		183	15.8	8.6	2.3	15	10.0	63	23	1.9	

[1] In experiments 3–14 each value represents the average of 2 rats whose thyroids and plasma were pooled for iodine analysis.

fed ad libitum and sacrificed on 1, 2, 3, 4, 9, 24 and 44 days after excision of their pituitary glands. In order to provide controls for the operated rats, each hypophysectomized rat was paired with a normal male rat of approximately equal weight. The daily food intake of each hypophysectomized rat that was permitted to survive 3 days or longer was measured and the amounts eaten fed to their respective normal controls on the next day. Thus, the iodine intake of the two groups was about the same.

RESULTS

Plasma Iodine—An effect of hypophysectomy upon plasma iodine was evident as early as 2 days later; at this time the values found for protein-bound iodine of plasma were about 20 per cent lower in the operated than in the control rats. In rats deprived of their pituitary gland for 3 days, a decrease of about 50 per cent occurred in both total and protein-bound iodine of plasma. No significant further drop in plasma iodine was observed in rats that were examined from 4 to 44 days after hypophysectomy.

In the dog, Houssay, Mazzocco, and Biasotti (1931) and Sturm (1934) reported that the total iodine level of the blood increases immediately after hypophysectomy, returns to normal at later intervals, and falls below normal several weeks or months after this operation. This response is quite different from that of the rat, which, as noted above, shows a lowered concentration in both total and protein-bound iodine of plasma as early as 3 days after excision of the hypophysis.

Unfortunately both Houssay *et al.* and Sturm determined total-blood iodine and not protein-bound iodine of plasma. The latter is a more reliable index of the level of circulating *thyroid hormone*. In the absence of such data it cannot be assumed that the thyroid hormone of the blood was increased in their dogs following hypophysectomy. Houssay *et al.* ascribed the increased level of blood iodine to a transitory hyperactivity of the thyroid, which they demonstrated histologically during the first few days after removal of the pituitary. Such hyperactivity has also been described by Dott (1923). Since the hyperactivity lasts only a few days, it is difficult to see how it can be responsible for an increased level of blood iodine that lasts for several months.

Thyroid Iodine—Hypophysectomy failed to decrease the amounts of thyroxine or of total iodine contained in the thyroid gland. A change in the size of the thyroid gland was detectable as early as two days after hypophysectomy, but at this interval the thyroxine and total iodine contained in the thyroid gland of the operated rats were identical with those of their unoperated controls. Between the intervals of 4 and 44 days after hypophysectomy, eight separate comparisions were made between hypophysectomized rats and their unoperated controls with respect to the content of thyroxine and total iodine of their thyroid glands. In six cases the amounts of thyroxine were greater in the hypophysectomized rats than in their controls; in the other two the amounts were about the same. In seven, the amounts of total iodine in the thyroid of the hypophysectomized rats were greater than in their respective controls. In keeping with the effect of hypophysectomy upon the size of the thyroid, there was a pronounced difference in these 2 groups of rats when the amounts of the iodine fractions were expressed on a percentage basis. Thus, 44 days after hypophysectomy, the thyroid glands of the operated and control rats

contained respectively 0.075 and 0.038 per cent total iodine and 0.023 and 0.012 per cent thyroxine iodine.

The plasma and thyroid glands of two rats[3] that had been deprived of their pituitary glands for more than one year were also examined. Even at this late interval after hypophysectomy, the amounts of thyroxine and total iodine contained in their thyroid glands did not differ from normal; the levels of iodine in their plasma, however, were still below the normal.

The effect of hypophysectomy upon the *total-iodine* content of the thyroid gland of another species, the dog, has been investigated by Houssay, Biasotti, and Mazzocco (1931) and by Hermann (1942). The former found that at intervals of 31 and 126 days after hypophysectomy the total-iodine contents of the gland were about the same as normal; this represents an increase, however, when expressed as a percentage of the weight of the thyroid tissue. This was confirmed by Hermann, who reported an average increase of 88 per cent in the concentration of total iodine in the glands of dogs 1–9 months after hypophysectomy. In the rats examined here the average increase in total-iodine concentration of the thyroid glands was 90 per cent.

Baumann, Metzer, and Marine (1942) determined the thyroxine and total iodine of the thyroids of 11 dogs that were subjected to hypophysectomy, only 2 of which showed a complete absence of the hypophysis at necropsy. They found normal amounts of thyroxine and total iodine in thyroid tissue that had been stored in formaldehyde for about a year before the analyses were carried out. Unfortunately the weights of the dogs were not given; hence it is difficult to assess the fact that they found the largest thyroid glands in the only 2 dogs that were completely hypophysectomized.

DISCUSSION

The observation that the level of protein-bound iodine in plasma falls as early as 48 hours after excision of the pituitary gland is not surprising in view of the well-known effects of hypophysectomy upon the morphology of the thyroid gland. The presence, however, of normal and even greater than normal amounts of thyroxine in the thyroid gland during the time that this is occurring throws new light on the mechanism by which thyroxine is released into the circulation by the gland. In the series of rats which were examined from 1 to 44 days after hypophysectomy, as well as in those examined as late as one year after hypophysectomy, the levels of protein-bound iodine of plasma were decreased by as much as 55 per cent despite the fact that the thyroxine content of the thyroid gland did not drop below normal in a single case. These findings leave no doubt that a lowered thyroxine content of plasma and peripheral tissues is not a *direct*

[3] We are indebted to Dr. Evelyn Anderson for making these animals available to us.

stimulus to the thyroid gland to release its thyroxine even when the gland contains greater amounts of thyroxine than normal. They also demonstrate quite clearly that, even in a gland that contains an amount of thyroxine greater than normal, its release into the blood stream requires the action of the thyrotropic hormone.

It is concluded that the thyrotropic hormone of the pituitary gland is concerned with at least two phases of thyroid function: (1) the rate of formation of thyroxine (Morton, Perlman, Anderson, and Chaikoff, 1942) and (2) the rate at which the thyroid hormone is released into the circulation.

SUMMARY

The level of protein-bound iodine of plasma is depressed by hypophysectomy. A decrease was observed as early as 48 hours. By the third day the concentration of protein-bound iodine of plasma was reduced by 50 per cent. No further drop occurred in rats that were examined as late as 44 days after hypophysectomy. Normal or greater than normal amounts of thyroxine are present in the thyroid glands of the hypophysectomized rat.

The role of the thyrotropic hormone in the rate of formation of thyroxine by the thyroid gland and in the release of thyroxine by the gland into the circulation is discussed.

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DISTURBANCES OF WATER METABOLISM IN VITAMIN DEFICIENCIES AND EFFECTS OF ADRENAL CORTICAL HORMONES[1]

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IT HAS BEEN REPORTED that pathological changes occur in the adrenal cortices of rats fed a diet deficient in pantothenic acid (Daft and Sebrell, 1939; Nelson, 1939; Daft, Sebrell, Babcock and Jukes, 1940; Ashburn, 1940; Salmon and Engel, 1940; Mills, Shaw, Elvehjem and Phillips, 1940). Adrenal cortical extract, desoxycorticosterone acetate and anterior pituitary extract fail to prevent adrenal hemorrhage in rats on such a deficient diet (Mushett and Unna, 1941). While it has been suggested, on a morphologic basis, that adrenal cortical insufficiency exists in these vitamin-deficient rats (Ashburn, 1940), no physiological evidence has been provided to indicate that the adrenal changes are associated with impairment of adrenal function. To gain at least suggestive evidence on this point a study was made of the response of animals on a pantothenic acid-deficient diet to water administered by stomach tube. The basis of these tests was the well-established finding that marked deficiencies in water diuresis and in resistance to water intoxication result from hypofunction of the adrenal cortex in animals and man.

In the course of these experiments it seemed advisable to compare results with those obtained in a vitamin deficiency in which no adrenal pathology was evident, and hence observations in riboflavin deficiency were included. It was found that the lack of either of these vitamins caused disturbances in certain aspects of water metabolism which resemble those seen in adrenal insufficiency and that these disturbances could be corrected by the administration of adrenal cortical hormones. An abstract on part of this work has been published (Gaunt, Liling and Mushett, 1945).

METHODS

Either 5 doses of water at hour intervals, or 13 doses at half-hour intervals were administered. Observations were made on the rate of diuresis

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and on the following indices of water intoxication: changes in body temperature, the frequency of collapse and convulsions, and mortality. In such experiments the resistance to water intoxication is not dependent entirely upon the rate of diuresis (Gaunt, Liling and Cordsen, 1945; Hays and Mathieson, 1945). Details of the procedure were all identical with those published previously (Gaunt, 1944; Liling and Gaunt, 1945) with the exception that in the present experiments each water dose was 5 cc. per 100 grams body weight. The deaths recorded in the table as "1st day" were those occurring within eight hours of the beginning of the experiment; those listed as "2nd day" occurred between the eighth and twenty-fourth hours.

The rats were started on the experimental diets at the age of three weeks (average weight 48 gms.). The pantothenic acid-deficient diet consisted of the following: vitamin-free casein (Labco), 24 per cent; sucrose, 60 per cent; salt mixture, U.S.P. XI No. 1, 4 per cent; crisco, 10 per cent; and cod liver oil 2 per cent. Of this diet 1 Kgm. was supplemented with 10 mgm. each of thiamine and pyridoxine, 20 mgm. riboflavin, 100 mgm. nicotinamide, 1 gm. choline chloride and 50 mgm. of inositol.

The riboflavin-deficient diet consisted of the following: vitamin-free casein (Labco), 18 per cent; dextrose, 68 per cent; salt mixture, U.S.P. XI No. 1, 4 per cent; crisco, 8 per cent and cod liver oil, 2 per cent. Of this diet 1 kgm. was supplemented with 8 mgm. each of thiamine and pyridoxine, 50 mgm. calcium pantothenate, 100 mgm. nicotinamide and 1 gm. of choline chloride.

After four to six weeks on these diets the animals were used for the water diuresis and tolerance studies. At this time all rats showed a marked retardation of growth; in fact, the weights of the riboflavin-deficient animals had become almost stationary. The pantothenic acid-deficient rats showed varying degrees of achromotrichia and alopecia was evident among the riboflavin-deficient rats. An occasional animal in both groups developed porphyrin encrustations on the whiskers.

Three types of control animals were used. a) One group was composed of animals of the same size but younger than the vitamin-deficient cases, fed a regular stock diet. b) Another consisted of pair-fed animals, of the same original lot as the vitamin-deficient cases, fed a purified diet adequately fortified with vitamin supplements. They were given this food, however, only in the limited amounts consumed by the vitamin-deficient rats. The growth rate of these animals, because of the reduced diet, had been severely curtailed. c) The third group were fed *ad libitum* the diet used in *b*.

These various groups of controls did not differ materially in their response to water and are averaged together in the tables. There is, however, a tendency for small rats in this size range to excrete water faster than larger ones, and hence the vitamin-deficient rats, being smaller than some of their controls, were given a slight "advantage" in the tests. This makes their deficiencies the more significant. For reasons discussed in previous papers all conclusions are based on comparisons between control and experimental animals tested simultaneously. In the riboflavin-deficient cases, where results were somewhat less consistent, the controls for each of the experimental groups are quoted separately in the tables.

RESULTS

Pantothenic acid deficiency. (Table 1, Series 1–4)

When given either 5 doses of water, which normally does not produce intoxication, or 13 doses which does produce intoxication, the various groups with pantothenic acid deficiency excreted water less rapidly than their various controls, and succumbed in much greater

TABLE I. AVERAGE FIGURES ON RESPONSE TO WATER INGESTION IN VITAMIN-DEFICIENT ANIMALS

Series No.	Treatment	No. rats used	Body Weight	Per cent water excreted at		Per cent deaths	
				3rd hr.	5th hr.	1st day	2nd day
	Pantothenic acid deficiency—Received 5 doses of water						
1	P.A. deficient—untreated	30	85	55	65	0	0
	Controls	12	114	79	78	0	0
	Pantothenic acid deficiency—Received 13 doses of water						
2	P.A. deficient—untreated	24	90	39	41	83	0
3	P.A. deficient Ca pantoth.	12	88	44	56	25	0
4	P.A. deficient Cort. Horm.	18	99	58	72	17	17
	Controls	25	120	46	53	35	21
	Riboflavin deficiency—Received 5 doses of water						
5	Riboflavin def.—untreated	18	63	49	53	17	11
6	Riboflavin def. 10γ ribo. daily	6	136	46	55	17	0
7[1]	Riboflavin def. 120γ ribo. daily	6	102	66	80	0	0
	Controls	18	73	75	80	0	0
	Riboflavin deficiency—Received 13 doses of water						
8	Riboflavin def.—untreated	16	65	39	45	62	13
	Controls	16	88	49	55	25	12
9	Riboflavin def.—selected cases	7	84	50	60	43	0
	Controls	7	93	60	63	29	0
10	Riboflavin def.—Cort. Hor.	10	82	63	68	33	33
	Controls	8	78	56	64	10	10

[1] The food intake of this group was restricted to that of group 5.

numbers from water intoxication. This deficiency was not of the striking sort seen after adrenalectomy for instance, but it was consistently observed in every group of animals, and was not dependent upon vitamin deficiency symptoms of a grossly severe nature. Collapse and convulsions were more frequent in the vitamin deficient cases; the characteristic temperature-reducing effects of excess water (Gaunt, 1944) were also more marked, but difficulty in making accu-

rate temperature readings in the smaller animals with our equipment precludes the emphasis previously given to such findings.

A normal diuresis and resistance to excess water could be restored in the vitamin-deficient cases by the administration of 1.0 mg. calcium pantothenate by stomach tube 18 hours before a water intoxication test, and the same dose repeated one-half hour before the test (Series 3).

Eighteen pantothenic acid deficient animals, given 13 doses of water, were treated with adrenal cortical hormones as follows: 1.5 mg. of DCA (desoxycorticosterone acetate[2]) was given in 0.15 cc. of peanut oil, 18 hours before the water tests, and the same dose repeated one-half hour before the test. In addition 0.5 cc. of adrenal cortical extract[3] was given with the third and sixth doses of water. Although this hormone dosage was relatively small, it exerted a striking protective action in vitamin-deficient animals, increasing in fact the rate of diuresis and number of survivors above that of normal controls (Series 4).

Riboflavin deficiency (Table 1, Series 5–10)

In average terms animals with riboflavin deficiency showed results of about the same kind as those with pantothenic acid deficiency. In the riboflavin-deficient series there were even some deaths from 5 doses of water which never occurs in normal animals. This effect, however, was not seen consistently, and some animals on the riboflavin-free diet were indistinguishable from normal ones. It was apparent that this variability was associated with the severity of the riboflavin deficiency symptoms in different cases, since all animals do not develop symptoms to the same degree after a given period on a vitamin-deficient diet. That this was actually the basis for the variability was shown in an experiment (Series 9) in which from a group of 30 riboflavin-deficient animals 7 were selected which grossly appeared to be in the best condition, i.e., the most free of deficiency symptoms. These selected animals were then compared with normal controls in response to 13 doses of water. If they handled water less well than the controls the differences were slight and not significant with the number of animals used. It is clear then that the abnormalities in response to water in riboflavin deficiency, unlike those in pantothenic acid deficiency, are terminal symptoms only.

The administration of 10 micrograms of riboflavin daily during the entire experimental period did not improve the response to 5 doses of water (Series 6), whereas the use of 120 micrograms daily maintained it at a normal level. (Series 7).

[2] The desoxycorticosterone was kindly supplied by Dr. H. W. Hays, Ciba Pharmaceutical Products, Inc.

[3] The adrenal cortical extract, a concentrated preparation, representing 250 grams adrenal tissue per cc. was made and generously supplied by Dr. W. W. Swingle, Princeton University.

One series of riboflavin-deficient animals were given cortical hormones in the same manner as described above in pantothenic acid deficiency. This therapy enabled water to be excreted at a normal rate, and reduced the number of deaths, particularly during the first day while the hormones were being given (Series 10).

DISCUSSION

It is apparent from these experiments that there is some deficiency in water metabolism which characterizes both of the avitaminotic states studied here. In riboflavin deficiency this is seen most clearly when the deficiency symptoms are severe; it is apparent in pantothenic acid deficiency when the symptoms are mild, and while the animals aside from reduced growth and achromatrichia appear to be "healthy." It is to be emphasized that deficiencies in handling water of the types seen here, while apparent in various conditions, are due to specific cause and are not characteristic of all states of general injury, debility, and weakness.

It was noted above for instance that the pair-fed controls, in a state of chronic inanition, responded to water normally. The same is true in hypothyroidism (Gaunt, Cordsen and Liling, 1945; Hays and Mathieson, personal communication); in chronic poisoning by the drug Germanin, provided the concomitant kidney damage is not excessive (Weiss and Gaunt, unpublished); in the poisoning resulting from the chronic, and eventually lethal, administration of formalin subcutaneously (Gaunt, unpublished); after removal of two-thirds of the liver (Gaunt and Liling, unpublished) and in a variety of clinical states observed by Robinson, Powers and Kepler (1941). In fact, the exposure of animals to stress may enhance the diuretic response to water and resistance to water intoxication (Liling and Gaunt, 1945), unless some specific damage interferes with renal, perhaps liver (Aldersberg and Fox, 1943), or adrenal cortical function.

Since adrenal cortical damage has been reported in pantothenic acid deficiency, and since the accompanying inefficiency in handling water is reparable by cortical hormones, there exists strong suggestive evidence of adrenal dysfunction in this state. Proof cannot be considered established, however, because the adrenal hormones not only restore the response to normal, but actually augment it. This indicates that a pharmacological overdosage action of these substances may be involved, and not simply the correction of pre-existing adrenal dysfunction.

There are no reports otherwise to suggest impairment of adrenal function in riboflavin deficiency and it is perhaps safest tentatively to regard the protective action of cortical hormones in this condition as a manifestation of their pharmacologic properties. Obviously the mechanism of the abnormalities in both of these avitaminotic conditions deserves further study.

Another aspect of the protective action of cortical hormones is of interest here. Such action of these substances against water intoxication has been previously demonstrated by many workers in adrenalectomized animals, in hypophysectomized animals (Gaunt, Remington and Schweizer, 1937; Joseph et al., 1945) and, after large doses, in normal animals as well (Gaunt, 1943). It is shown here that this protective action can be seen in certain abnormal conditions, even with relatively small doses. It would appear then that among the few types of lethal stress against which the cortical hormones are protective (Swingle and Remington, 1944), that involving water intoxication is one of the most sensitive, considering the ease with which it can be demonstrated and its independence of the condition of the animal.

SUMMARY

Rats on a diet deficient in pantothenic acid show a lethargic diuresis when water is given by stomach tube, and a decreased resistance to water intoxication. These defects are remedied by the administration of either calcium pantothenate or adrenal cortical hormones. In view of the reported adrenal pathology in pantothenic acid deficiency, the results are consistent with but do not prove the idea that adrenal hypofunction exists in this vitamin deficiency.

A similar inability to handle water normally is seen in riboflavin deficiency, but only during the later stages when deficiency symptoms are severe. The prophylactic use of riboflavin prevented these abnormal responses to water, and cortical hormones, given only at the time tests were run, had the same effect.

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NOTES AND COMMENTS

ESTROGEN INACTIVATION AND FOLIC ACID

Recently the importance of folic acid (L. casei factor) for different functions has been studied.

Hertz reported a quantitative relation between the influence of stilbestrol on the oviduct and folic acid. This latter substance seems indispensable for the full reaction. Leuchtenberger et al. have described a tumor inhibiting influence of folic acid. As folic acid is to be found in the liver and liver inactivates estrogens, it seemed of some interest to study the influence of folic acid on estrogens.

As folic acid was not soluble in water, we made all our following experiments with a solution of crystalline folic acid[1] in N/100 NaOH. The final liquid had a pH about 8.5. Several different series of combinations incubating folic acid with estrone at 37° C. were run.

To a solution of 10 milligrams of folic acid in 100 cc. of N/100 NaOH, 4 cc. of an alcoholic solution containing 1000 international units (0, 1 mg.) of estrone were added and allowed to stay for 18 hours at 37°. The liquid was then assayed on spayed white mice.

The same combination of folic acid and estrone was also assayed after

TABLE 1

Assayed	Incubation time (hrs.)	Number of animals	Units of estrogen assayed	Results	
				positive	negative
Folic acid + estrogen	18	5	10	2	3
		5	5	0	5
		5	2, 5	0	5
Folic acid + estrogen	8	3	10	3	0
		3	5	3	0
		3	2, 5	0	3
Folic acid + estrogen	2	5	10	5	0
		5	5	2	3
		5	2, 5	0	5
Folic acid + estrogen	0	3	10	2	1
		3	5	2	1
		3	2, 5	1	2
Controls		3	10	3	0
		3	5	3	0
		15	2, 5	15	0

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[1] The folic acid (L. Casei factor) was obtained thorugh the courtesy of Dr. E. L. R. Stokstad of Lederle Laboratories Inc.

8 hours, or two hours incubation at 37°, and also immediately after having been mixed together. For controls, equal doses of estrone which had been kept for various times at the same temperature with N/100 NaOH were injected into other white mice.

The results of these experiments are summarized in Table 1.

In a former paper one of us together with Rosenberg reported, that the estrone inactivating principle can be extracted from the liver.

Nine hundred grams of fresh beef liver were allowed to stay with 900 cc. of distilled water at pH 3 for 24 hours, filtered, and neutralized. An amount corresponding to 1 gm. of fresh liver inactivated 5 I.U. of estrone. Mixtures of this extract were assayed together with our folic acid solution and estrone. There was no mutual influence, the inactivating power of the liver extract was neither increased not inhibited by folic acid.

In résumé we can say that folic acid in relatively high amounts inhibits the estrogenic activity of estrone to a certain, rather moderate degree. One milligram of folic acid inactivates about 50 I.U. of estrone. Folic acid is certainly not the substance responsible for the vigorous estrogen inactivating potency of liver in vitro.

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NOTICE

AMERICAN SOCIETY FOR RESEARCH IN PSYCHOSOMATIC PROBLEMS

714 Madison Ave., New York 21, N. Y.

ANNUAL MEETING

Hotel Pennsylvania, New York City
May 11, 1946

A.M.

Contributions of Military Medicine to Psychosomatic Medicine

P.M.

Psychosomatic Aspects of Orthopedic Practice

ANNUAL DINNER

"New Advances in Psychosomatic Investigative Techniques"
(An Illustrated Parody)
Bertram D. Lewin, M.D.

May 12, 1946
Submitted Papers

Because of space limitation, reservations should be made at least 2 weeks prior to meeting. Registration fee for non-members is $5.00 for two days; $3.00 for one. The charge for the Annual Dinner is $5.00. Limited hotel reservations are available.

NEW BOOK

BIOENERGETICS AND GROWTH. By Samuel Brody, Ph.D., Reinhold Publishing Corp., New York, 1945, Price $8.50.

The phenomenon of normal growth in man and other animals is of constant interest to the endocrinologist. A consideration of the many aspects involved in the growth of the organism is set forth in Brody's "Bioenergetics and Growth."

The author is a member of the staff of the Missouri Agricultural Experiment Station. His long-time interest in mathematical analysis of growth, particularly in domestic animals as set forth in many publications, is well known to investigators in this field.

This volume is an attempt to integrate many of the isolated concepts affecting growth into a formulation of the basic physiological principles involved. There are twenty-five chapters devoted to this task, the titles of which are frequently more inviting than the information is satisfying. The chapter headings include, among others: "Energetic efficiencies of growth and work process (Chapt. 3); Metabolic catalysts in the efficiency complex: enzymes, minerals and vitamins in biological oxidations (Chapt. 6); Homeostasis and organismic theory (Chapt. 10); Linear growth, form and function (Chapt. 17)."

Chapter 7 is entitled "Metabolic catalysts in the efficiency complex: Hormones." In these 65 pages the author has attempted to indicate the role of all of the hormones in the processes of growth and maturation. While there are no substantial errors of omission nor of commission, this chapter contains only statements of fact supported by abundant citations from the literature, and as an integrated review is not as competent as other recent critical summaries. It can only be hoped that at another time a closer integration of hormones, vitamins and other catalytic agents will be effected with the "energetics" under discussion.

In a later section a well-documented and critical account of temperature in the life processes is a significant contribution and appears to be an important and useful summary.

The author's long experience in the use of mathematical forms of expression is reflected in several chapters which constitute the most scholarly and important sections of material presented. These analyses of growth rates, of linear growth and of certain aspects of ageing and efficiency are exceedingly well done.

This is a unique book which cannot be read casually, but which should be at hand on the bookshelf for ready reference.

It is not a perfect resume of the literature. It certainly lacks integration in the discussions of the manifold aspects of growth and metabolism. However, this book may be only the first attempt at such an integration. It is possible that the subject cannot be fully treated by a single author. The subject is so complex that an analysis by many authors may be the best solution.

The author should not be adversely criticized, but should be highly commended for having attempted a most difficult task. Dr. Brody has produced a book which is oriented in the right direction and which will be the object of much careful study and discussion. In the opinion of the present reviewer, the volume stands alone and must serve as a useful and necessary compendium until someone at a later time writes a better treatise on the dynamics of bioenergetics and growth.

E. T. ENGLE